WO9833919

Publication Title:

ANTI-ALPHAVBETA3 RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS
ENCODING SAME AND METHODS OF USE

Abstract:

The invention provides a Vitaxin antibody and a LM609 grafted antibody
exhibiting selective binding affinity to alphavbeta3. The Vitaxin antibody consists
of at least one Vitaxin heavy chain polypeptide and at least one Vitaxin light
chain polypept
5f8
ide or functional fragments thereof. Also provided are the Vitaxin heavy and light
chain polypeptides and functional fragments. The LM609 grafted antibody
consists of at least one CDR grafted heavy chain polypeptide and at least one
CDR grafted light chain polypeptide or functional fragment thereof. Nucleic acids
encoding Vitaxin and LM609 grafted heavy and light chains as well as nucleic
acids encoding the parental non-human antibody LM609 are additionally
provided. Functional fragments of such encoding nucleic acids are similarly
provided. The invention also provides a method of inhibiting a function of
alphavbeta3. The method consists of contacting alphavbeta3 with Vitaxin or a
LM609 grafted antibody or functional fragments thereof under conditions which
allow binding to alphavbeta3. Finally, the invention provides for a method of
treating an alphavbeta3-mediated disease. The method consists of administering
an effective amount of Vitaxin or a LM609 grafted antibody or functional fragment
thereof under conditions which allow binding to alphavbeta3.

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{6} : C12N 15/13, 15/62, C07K 16/28, 16/46, A61K 39/395	**A2**	(11) International Publication Number: **WO 98/33919** (43) International Publication Date: 6 August 1998 (06.08.98)

(21) International Application Number: PCT/US98/01826

(22) International Filing Date: 30 January 1998 (30.01.98)

(30) Priority Data:
    08/791,391   30 January 1997 (30.01.97)   US

(71) Applicant: IXSYS, INCORPORATED [US/US]; 3520 Dunhill Road, San Diego, CA 92121 (US).

(72) Inventors: HUSE, William, D.; 471 Avenida Primavera, Del Mar, CA 92014 (US). GLASER, Scott, M.; 3026 36th Avenue S.W., Seattle, WA 98126 (US).

(74) Agents: GAY, David, A. et al.; Campbell & Flores LLP, Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ANTI–ALPHA–V BETA–3 RECOMBINANT HUMANIZED ANTIBODIES, NUCLEIC ACIDS ENCODING SAME AND METHODS OF USE

(57) Abstract

    The invention provides a Vitaxin antibody and a LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$. The Vitaxin antibody consists of at least one Vitaxin heavy chain polypeptide and at least one Vitaxin light chain polypeptide or functional fragments thereof. Also provided are the Vitaxin heavy and light chain polypeptides and functional fragments. The LM609 grafted antibody consists of at least one CDR grafted heavy chain polypeptide and at least one CDR grafted light chain polypeptide or functional fragment thereof. The invention additionally provides a high affinity LM609 grafted antibody comprising one or more CDRs having at least one amino acid substitution, where the $\alpha_v\beta_3$ binding activity of the high affinity LM609 grafted antibody is enhanced. Nucleic acids encoding Vitaxin and LM609 grafted heavy and light chains as well as nucleic acids encoding the parental non–human antibody LM609 are additionally provided. Functional fragments of such encoding nucleic acids are similarly provided. The invention also provides a method of inhibiting a function of $\alpha_v\beta_3$. The method consists of contacting $\alpha_v\beta_3$ with Vitaxin or a LM609 grafted antibody or functional fragments thereof under conditions which allow binding to $\alpha_v\beta_3$. Finally, the invention provides for a method of treating an $\alpha_v\beta_3$–mediated disease. The method consists of administering an effective amount of Vitaxin or a LM609 grafted antibody or functional fragment thereof under conditions which allow binding to $\alpha_v\beta_3$.

ANTI–ALPHA–V BETA–3 RECOMBINANT HUMANIZED ANTIBODIES, NUCLEIC ACIDS ENCODING SAME AND METHODS OF USE

This application is a continuation-in-part of United States Serial No. 08/791,391, filed January 30, 5 1997, the entire contents of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates generally to integrin mediated diseases and, more particularly, to 10 nucleic acids encoding $\alpha_v\beta_3$-inhibitory monoclonal antibodies and to CDR grafted $\alpha_v\beta_3$-inhibitory antibodies for the therapeutic treatment of $\alpha_v\beta_3$-mediated diseases.

Integrins are a class of cell adhesion receptors that mediate both cell-cell and cell-15 extracellular matrix adhesion events. Integrins consist of heterodimeric polypeptides where a single α chain polypeptide noncovalently associates with a single β chain. There are now about 14 distinct α chain polypeptides and at least about 8 different β chain 20 polypeptides which constitute the integrin family of cell adhesion receptors. In general, different binding specificities and tissue distributions are derived from unique combinations of the α and β chain polypeptides or integrin subunits. The family to which a particular 25 integrin is associated with is usually characterized by the β subunit. However, the ligand binding activity of the integrin is largely influenced by the α subunit. For example, vitronectin binding integrins contain the α_v integrin subunit.

2

It is now known that the vitronectin binding integrins consist of at least three different α_v containing integrins. These α_v containing integrins include $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$, all of which exhibit different

5 ligand binding specificities. For example, in addition to vitronectin, $\alpha_v\beta_3$ binds to a large variety of extracellular matrix proteins including fibronectin, fibrinogen, laminin, thrombospondin, von Willebrand factor, collagen, osteopontin and bone sialoprotein I.

10 The integrin $\alpha_v\beta_1$ binds to fibronectin, osteopontin and vitronectin whereas $\alpha_v\beta_5$ is known to bind to vitronectin and osteopontin.

As cell adhesion receptors, integrins are involved in a variety of physiological processes

15 including, for example, cell attachment, cell migration and cell proliferation. Different integrins play different roles in each of these biological processes and the inappropriate regulation of their function or activity can lead to various pathological conditions.

20 For example, inappropriate endothelial cell proliferation during neovascularization of a tumor has been found to be mediated by cells expressing vitronectin binding integrins. In this regard, the inhibition of the vitronectin-binding integrin $\alpha_v\beta_3$ also inhibits this

25 process of tumor neovascularization. By this same criteria, $\alpha_v\beta_3$ has also been shown to mediate the abnormal cell proliferation associated with restenosis and granulation tissue development in cutaneous wounds, for example. Additional diseases or pathological states

30 mediated or influenced by $\alpha_v\beta_3$ include, for example, metastasis, osteoporosis, age-related macular degeneration and diabetic retinopathy, and inflammatory diseases such as rheumatoid arthritis and psoriasis. Thus, agents which can specifically inhibit vitronectin-

3

binding integrins would be valuable for the therapeutic
treatment of diseases.

 5   Many integrins mediate their cell adhesive
functions by recognizing the tripeptide sequence Arg-Gly-
Asp (RGD) found within a large number of extracellular
matrix proteins. A variety of approaches have attempted
to model agents after this sequence to target a
particular integrin-mediated pathology. Such approaches
include, for example, the use of RGD-containing peptides
10   and peptide analogues which rely on specificity to be
conferred by the sequences flanking the RGD core
tripeptide sequence. Although there has been some
limited success, most RGD-based inhibitors have been
shown to be, at most, selective for the targeted integrin
15   and therefore exhibit some cross-reactivity to other
non-targeted integrins. Such cross-reactive inhibitors
therefore lack the specificity required for use as an
efficacious therapeutic. This is particularly true for
previously identified inhibitors of the integrin $\alpha_v\beta_3$.

20   Monoclonal antibodies on the other hand exhibit
the specificity required to be used as an effective
therapeutic. Antibodies also have the advantage in that
they can be routinely generated against essentially any
desired antigen. Moreover, with the development of
25   combinatorial libraries, antibodies can now be produced
faster and more efficiently than by previously used
methods within the art. The use of combinatorial
methodology also allows for the selection of the desired
antibody along with the simultaneous isolation of the
30   encoding heavy and light chain nucleic acids. Thus,
further modification can be performed to the
combinatorial antibody without the incorporation of an
additional cloning step.

4

Regardless of the potential advantages associated with the use of monoclonal antibodies as therapeutics, these molecules nevertheless have the drawback in that they are almost exclusively derived from
5   non-human mammalian organisms. Therefore, their use as therapeutics is limited by the fact that they will normally elicit a host immune response. Methods for substituting the antigen binding site or complementarity determining regions (CDRs) of the non-human antibody into
10 a human framework have been described. Such methods vary in terms of which amino acid residues should be substituted as the CDR as well as which framework residues should be changed to maintain binding specificity. In this regard, it is understood that
15 proper orientation of the β sheet architecture, correct packing of the heavy and light chain interface and appropriate conformation of the CDRs are all important for preserving antigen specificity and affinity within the grafted antibody. However, all of these methods
20 require knowledge of the nucleotide and amino acid sequence of the non-human antibody and the availability of an appropriately modeled human framework.

Thus, there exists a need for the availability of nucleic acids encoding integrin inhibitory antibodies
25 which can be used as compatible therapeutics in humans. For $\alpha_v\beta_3$-mediated diseases, the present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

30   The invention provides a Vitaxin antibody and a LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$. The Vitaxin antibody consists of at

5

least one Vitaxin heavy chain polypeptide and at least
one Vitaxin light chain polypeptide or functional
fragments thereof. Also provided are the Vitaxin heavy
and light chain polypeptides and functional fragments.

5   The LM609 grafted antibody consists of at least one LM609
CDR grafted heavy chain polypeptide and at least one
LM609 CDR grafted light chain polypeptide or functional
fragment thereof. The invention additionally provides a
high affinity LM609 grafted antibody comprising one or

10 more CDRs having at least one amino acid substitution,
where the $\alpha_v\beta_3$ binding activity of the high affinity LM609
grafted antibody is enhanced. Nucleic acids encoding
Vitaxin and LM609 grafted heavy and light chains as well
as nucleic acids encoding the parental non-human antibody

15 LM609 are additionally provided. Functional fragments of
such encoding nucleic acids are similarly provided. The
invention also provides a method of inhibiting a function
of $\alpha_v\beta_3$. The method consists of contacting $\alpha_v\beta_3$ with
Vitaxin or a LM609 grafted antibody or functional

20 fragments thereof under conditions which allow binding to
$\alpha_v\beta_3$. Finally, the invention provides for a method of
treating an $\alpha_v\beta_3$-mediated disease. The method consists of
administering an effective amount of Vitaxin or a LM609
grafted antibody or functional fragment thereof under

25 conditions which allow binding to $\alpha_v\beta_3$.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide and deduced amino
acid sequence of the variable region of the antibody
Vitaxin. Figure 1A shows the nucleotide and deduced

30 amino acid sequences for the Vitaxin heavy chain variable
region (Gln1-Ser117; SEQ ID NOS:1 and 2, respectively)
while Figure 1B shows the nucleotide and deduced amino

6

acid sequences for the Vitaxin light chain variable

region (Glu1-Lys107; SEQ ID NOS:3 and 4, respectively).

Figure 2 shows the nucleotide and deduced amino

acid sequence of the variable region of the monoclonal

5   antibody LM609. Figure 2A shows the nucleotide and

deduced amino acid sequence of the LM609 heavy chain

variable region (SEQ ID NOS:5 and 6, respectively). The

variable region extends from amino acid Glu1 to Ala117.

Figure 2B shows the nucleotide and deduced amino acid

10   sequence of the LM609 light chain variable region (SEQ ID

NOS:7 and 8, respectively). The variable region of the

light chain extends from amino acid Asp1 to Lys107.

Figure 3 shows the competitive inhibition of

LM609 IgG binding to the integrin $_{avb3}$ with recombinant

15   LM609 Fab. Soluble recombinant murine LM609 Fab

fragments were prepared from periplasmic fractions of M13

bacteriophage clones muLM609M13 12 and muLM609M13 29.

The periplasm samples were serially diluted, mixed with

either 1 ng/ml, 5 ng/ml, or 50 ng/ml of LM609 IgG and

20   then incubated in 96 well plates coated with purified

$\alpha_v\beta_3$. Plates were washed and bound LM609 IgG detected

with goat anti-murine Fc specific antibody conjugated to

alkaline phosphatase. Fab produced by clone muLM609M13

12 inhibits both 1 ng/ml and 5 ng/ml LM609 IgG binding at

25   all concentrations of Fab greater than 1:27 dilution.

Figure 4 shows the characterization of Vitaxin

binding specificity. Figure 4A shows specific binding of

Vitaxin to the integrin $\alpha_v\beta_3$ compared to integrins $\alpha_{IIb}\beta_3$

and $\alpha_v\beta_5$. Figure 4B shows the competitive inhibition of

30   LM609 binding to $\alpha_v\beta_3$ by Vitaxin. Figure 4C shows the

competitive inhibition of fibrinogen binding to $\alpha_v\beta_3$ by

Vitaxin.

7

Figure 5 shows the inhibition of $\alpha_v\beta_3$-mediated cell attachment (5A) and migration (5B) by Vitaxin.

Figure 6 shows the reduction in tumor growth due to Vitaxin mediated inhibition of neovascularization. Figure 6A shows the inhibition of the $\alpha_v\beta_3$-negative Fg and HEp-3 human tumor fragments grown on chick chorioallantoic membranes (CAMs) following Vitaxin treatment. Figure 6B shows the growth inhibition of Vx2 carcinomas implanted subcutaneously in rabbits at two different Vitaxin doses administered 1 day post implantation. Figure 6C similarly shows Vx2 tumor growth inhibition as in Figure 6B, except that four different Vitaxin doses were administered beginning at 7 days post implantation.

Figure 7 shows the nucleotide and deduced amino acid sequence of the light chain variable region of the LM609 grafted antibody fragment (Glu1-Lys107; SEQ ID NOS:31 and 32, respectively). Position 49 of the light chain variable region can at least be either Arg or Met. The nucleotide and deduced amino acid sequence of the heavy chain variable region of the LM609 grafted antibody fragment is shown in Figure 1A (SEQ ID NOS:1 and 2, respectively).

Figure 8 shows the titration of LM609 grafted antibody variants and LM609 grafted Fab on immobilized $\alpha_v\beta_3$. Bacterial cell lysates containing LM609 grafted antibody (closed circles), LM609 grafted antibody variants with improved affinity isolated from the primary libraries (S102, closed squares; Y100, open squares; and Y101, open triangles) or from combinatorial libraries (closed triangles), or an irrelevant Fab (open circles) were titrated on immobilized $\alpha_v\beta_3$.

8

Figure 9 shows the construction of combinatorial libraries of enhanced LM609 grafted antibody variants containing multiple amino acid substitutions.

5   Figure 10 shows the inhibition of fibrinogen binding to $\alpha_V\beta_3$ by LM609 grafted antibody variants. Figure 10A shows inhibition of fibrinogen binding to immobilized $\alpha_V\beta_3$. Figure 1B shows correlation of affinity of antibody variants with inhibition of fibrinogen
10 binding.

Figure 11 shows the inhibition of M21 human melanoma cell adhesion to fibrinogen by LM609 grafted antibody variants. Cell binding to 10 μg/ml fibrinogen-coated substrate was assessed in the presence of various
15 concentrations of LM609 grafted Fab (closed triangles) or the enhanced LM609 grafted Fabs S102 (open circles), G102 (closed circles), or C37 (open triangles).

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to nucleic acids
20 encoding the monoclonal antibody (MAb) LM609. This antibody specifically recognizes the integrin $\alpha_V\beta_3$ and inhibits its functional activity. The invention is also directed to nucleic acids encoding and to polypeptides comprising non-murine grafted forms of LM609. These
25 grafted antibodies retain the binding specificity and inhibitory activity of the parent murine antibody LM609. The invention is additionally directed to optimized forms of LM609 grafted antibodies that exhibit increased binding affinity and specificity compared to the
30 non-mouse parental forms of the LM609 grafted antibody.

9

        In one embodiment, the hybridoma expressing
LM609 was used as a source to generate and clone cDNAs
encoding LM609. The heavy and light chain encoding cDNAs
were sequenced and their CDR regions were substituted
5   into a human antibody framework to generate the
non-murine form of the antibody. The substitution or
grafting of the CDRs was performed by codon-based
mutagenesis to generate a combinatorial antibody Fab
library consisting of members that presented alternative
10 residues at certain positions. Screening of the library
resulted in the isolation of Vitaxin. As a grafted
antibody containing human framework sequences, it is
unlikely that Vitaxin will elicit a host immune response
and can therefore be advantageously used for the
15 treatment of $\alpha_v\beta_3$-mediated diseases.

        As used herein, the term "monoclonal antibody
LM609" or "LM609" is intended to mean the murine
monoclonal antibody specific for the integrin $\alpha_v\beta_3$ which
is described by Cheresh, D.A. Proc. Natl. Acad. Sci. USA
20 84:6471-6475 (1987) and by Cheresh and Spiro J. Biol.
Chem. 262:17703-17711 (1987). LM609 was produced against
and is reactive with the M21 cell adhesion receptor now
known as the integrin $\alpha_v\beta_3$. LM609 inhibits the attachment
of M21 cells to $\alpha_v\beta_3$ ligands such as vitronectin,
25 fibrinogen and von Willebrand factor (Cheresh and Spiro,
supra) and is also an inhibitor of $\alpha_v\beta_3$-mediated
pathologies such as tumor induced angiogenesis (Brooks et
al. Cell 79:1157-1164 (1994), granulation tissue
development in cutaneous wound (Clark et al., Am. J.
30 Pathology, 148:1407-1421 (1996)) and smooth muscle cell
migration such as that occurring during restenosis (Choi
et al., J. Vascular Surg., 19:125-134 (1994); Jones et
al., Proc. Natl. Acad. Sci. 93:2482-2487 (1996)).

10

As used herein, the term "Vitaxin" is intended
to refer to a non-mouse antibody or functional fragment
thereof having substantially the same heavy and light
chain CDR amino acid sequences as found in LM609. The
5   term "Vitaxin" when used in reference to heavy or light
chain polypeptides is intended to refer to a non-mouse
heavy or light chain or functional fragment thereof
having substantially the same heavy or light chain CDR
amino acid sequences as found in the heavy or light chain
10   of LM609, respectively. When used in reference to a
functional fragment, not all LM609 CDRs need to be
represented. Rather, only those CDRs that would normally
be present in the antibody portion that corresponds to
the functional fragment are intended to be referenced as
15   the LM609 CDR amino acid sequences in the Vitaxin
functional fragment. Similarly, the use of the term
"Vitaxin" in reference to an encoding nucleic acid is
intended to refer to a nucleic acid encoding a non-mouse
antibody or functional fragment having substantially the
20   same nucleotide sequence as the heavy and light chain CDR
nucleotide sequences and encoding substantially the same
CDR amino acid sequences as found in LM609.

As used herein, the term "LM609 grafted
antibody" is intended to refer to a non-mouse antibody or
25   functional fragment thereof having substantially the same
heavy and light chain CDR amino acid sequences as found
in LM609 and absent of the substitution of LM609 amino
acid residues outside of the CDRs as defined by Kabat et
al., U.S. Dept. of Health and Human Services, "Sequences
30   of Proteins of Immunological Interest" (1983). The term
"LM609 grafted antibody" or "LM609 grafted" when used in
reference to heavy or light chain polypeptides is
intended to refer to a non-mouse heavy or light chain or
functional fragment thereof having substantially the same

11

heavy or light chain CDR amino acid sequences as found in
the heavy or light chain of LM609, respectively, and also
absent of the substitution of LM609 residues outside of
the CDRs as defined by Kabat et al., *supra*. When used in
5   reference to a functional fragment, not all LM609 CDRs
need to be represented. Rather, only those CDRs that
would normally be present in the antibody portion that
corresponds to the functional fragment are intended to be
referenced as the LM609 CDR amino acid sequences in the
10   LM609 grafted functional fragment. Similarly, the term
"LM609 grafted antibody" or "LM609 grafted" used in
reference to an encoding nucleic acid is intended to
refer to a nucleic acid encoding a non-mouse antibody or
functional fragment being absent of the substitution of
15   LM609 amino acids outside of the CDRs as defined by Kabat
et al., *supra* and having substantially the same
nucleotide sequence as the heavy and light chain CDR
nucleotide sequences and encoding substantially the same
CDR amino acid sequences as found in LM609 and as defined
20   by Kabat et al., *supra*.

The term "grafted antibody" or "grafted" when
used in reference to heavy or light chain polypeptides or
functional fragments thereof is intended to refer to a
heavy or light chain or functional fragment thereof
25   having substantially the same heavy or light chain CDR of
a donor antibody, respectively, and also absent of the
substitution of donor amino acid residues outside of the
CDRs as defined by Kabat et al., *supra*. When used in
reference to a functional fragment, not all donor CDRs
30   need to be represented. Rather, only those CDRs that
would normally be present in the antibody portion that
corresponds to the functional fragment are intended to be
referenced as the donor CDR amino acid sequences in the
functional fragment. Similarly, the term "grafted

12

antibody" or "grafted" when used in reference to an
encoding nucleic acid is intended to refer to a nucleic
acid encoding an antibody or functional fragment, being
absent of the substitution of donor amino acids outside
5   of the CDRs as defined by Kabat et al., *supra* and having
substantially the same nucleotide sequence as the heavy
and light chain CDR nucleotide sequences and encoding
substantially the same CDR amino acid sequences as found
in the donor antibody and as defined by Kabat et al.,
10   *supra*.

The meaning of the above terms are intended to
include minor variations and modifications of the
antibody so long as its function remains uncompromised.
Functional fragments such as Fab, $F(ab)_2$, Fv, single chain
15   Fv (scFv) and the like are similarly included within the
definition of the terms LM609 and Vitaxin. Such
functional fragments are well known to those skilled in
the art. Accordingly, the use of these terms in
describing functional fragments of LM609 or the Vitaxin
20   antibody are intended to correspond to the definitions
well known to those skilled in the art. Such terms are
described in, for example, Harlow and Lane, Antibodies: A
Laboratory Manual, Cold Spring Harbor Laboratory, New
York (1989); Molec. Biology and Biotechnology: A
25   Comprehensive Desk Reference (Myers, R.A. (ed.), New
York: VCH Publisher, Inc.); Huston et al., Cell
Biophysics, 22:189-224 (1993); Plückthun and Skerra,
Meth. Enzymol., 178:497-515 (1989) and in Day, E.D.,
Advanced Immunochemistry, Second Ed., Wiley-Liss, Inc.,
30   New York, NY (1990).

As with the above terms used for describing
functional fragments of LM609, Vitaxin and a LM609
grafted antibody, the use of terms which reference other

13

LM609, Vitaxin or LM609 grafted antibody domains,
functional fragments, regions, nucleotide and amino acid
sequences and polypeptides or peptides, is similarly
intended to fall within the scope of the meaning of each
5   term as it is known and used within the art. Such terms
include, for example, "heavy chain polypeptide" or "heavy
chain", "light chain polypeptide" or "light chain",
"heavy chain variable region" (V_H) and "light chain
variable region" (V_L) as well as the term "complementarity
10   determining region" (CDR).

In the case where there are two or more
definitions of a term which is used and/or accepted
within the art, the definition of the term as used herein
is intended to include all such meanings unless
15   explicitly stated to the contrary. A specific example is
the use of the term "CDR" to describe the non-contiguous
antigen combining sites found within the variable region
of both heavy and light chain polypeptides. This
particular region has been described by Kabat et al.,
20   *supra*, and by Chothia et al., <u>J. Mol. Biol.</u> 196:901-917
(1987) and by MacCallum et al., <u>J. Mol. Biol.</u> 262:732-745
(1996) where the definitions include overlapping or
subsets of amino acid residues when compared against each
other. Nevertheless, application of either definition to
25   refer to a CDR of LM609, Vitaxin, LM609 grafted
antibodies or variants thereof is intended to be within
the scope of the term as defined and used herein. The
amino acid residues which encompass the CDRs as defined
by each of the above cited references are set forth below
30   in Table 1 as a comparison.

14

Table 1: CDR Definitions

	Kabat[1]	Chothia[2]	MacCallum[3]
V_H CDR1	31-35	26-32	30-35
V_H CDR2	50-65	53-55	47-58
V_H CDR3	95-102	96-101	93-101
V_L CDR1	24-34	26-32	30-36
V_L CDR2	50-56	50-52	46-55
V_L CDR3	89-97	91-96	89-96

[1] Residue numbering follows the nomenclature of Kabat et al., *supra*

[2] Residue numbering follows the nomenclature of Clothia et al., *supra*

[3] Residue numbering follows the nomenclature of MacCallum et al., *supra*

As used herein, the term "substantially" or "substantially the same" when used in reference to a nucleotide or amino acid sequence is intended to mean that the nucleotide or amino acid sequence shows a considerable degree, amount or extent of sequence identity when compared to a reference sequence. Such considerable degree, amount or extent of sequence identity is further considered to be significant and meaningful and therefore exhibit characteristics which are definitively recognizable or known. Thus, a nucleotide sequence which is substantially the same nucleotide sequence as a heavy or light chain of LM609, Vitaxin, or a LM609 grafted antibody including fragments thereof, refers to a sequence which exhibits characteristics that are definitively known or recognizable as encoding or as being the amino acid sequence of LM609, Vitaxin or a LM609 grafted antibody. Minor modifications thereof are included so long as they

15

are recognizable as a LM609, Vitaxin or a LM609 grafted
antibody sequence. Similarly, an amino acid sequence
which is substantially the same amino acid sequence as a
heavy or light chain of Vitaxin, a LM609 grafted antibody

5   or functional fragment thereof, refers to a sequence
which exhibits characteristics that are definitively
known or recognizable as representing the amino acid
sequence of Vitaxin or a LM609 grafted antibody and minor
modifications thereof.

10   When determining whether a nucleotide or amino
acid sequence is substantially the same as Vitaxin or a
LM609 grafted antibody, consideration is given to the
number of changes relative to the Vitaxin or LM609
grafted antibody together with whether the function is

15 maintained. For example, a single amino acid change in a
3 amino acid CDR or several changes in a 16 amino acid
CDR are considered to be substantially the same if $\alpha_v\beta_3$
binding function is maintained. Thus, a nucleotide or
amino acid sequence is substantially the same if it

20 exhibits characteristics that are definitively known or
recognizable as representing the nucleotide or amino acid
sequence of Vitaxin or a LM609 grafted antibody and minor
modifications thereof as long as Vitaxin or LM609 grafted
antibody function is maintained.

25   As used herein, the term "fragment" when used
in reference to a nucleic acid encoding LM609, Vitaxin or
a LM609 grafted antibody is intended to mean a nucleic
acid having substantially the same sequence as a portion
of a nucleic acid encoding LM609, Vitaxin or a LM609

30 grafted antibody. The nucleic acid fragment is
sufficient in length and sequence to selectively
hybridize to an LM609, a Vitaxin or a LM609 grafted
antibody encoding nucleic acid or a nucleotide sequence

16

that is complementary to an LM609, Vitaxin or LM609 grafted antibody encoding nucleic acid. Therefore, fragment is intended to include primers for sequencing and polymerase chain reaction (PCR) as well as probes for

5 nucleic acid blot or solution hybridization. The meaning of the term is also intended to include regions of nucleotide sequences that do not directly encode LM609 polypeptides such as the introns, and the untranslated region sequences of the LM609 encoding gene.

10   As used herein, the term "functional fragment" when used in reference to Vitaxin, to a LM609 grafted antibody or to heavy or light chain polypeptides thereof is intended to refer to a portion of Vitaxin or a LM609 grafted antibody including heavy or light chain

15 polypeptides which still retains some or all or the $\alpha_v\beta_3$ binding activity, $\alpha_v\beta_3$ binding specificity and/or integrin $\alpha_v\beta_3$-inhibitory activity. Such functional fragments can include, for example, antibody functional fragments such as Fab, F(ab)$_2$, Fv, single chain Fv (scFv). Other

20 functional fragments can include, for example, heavy or light chain polypeptides, variable region polypeptides or CDR polypeptides or portions thereof so long as such functional fragments retain binding activity, specificity or inhibitory activity. The term is also intended to

25 include polypeptides encompassing, for example, modified forms of naturally occurring amino acids such as D-stereoisomers, non-naturally occurring amino acids, amino acid analogues and mimetics so long as such polypeptides retain functional activity as defined above.

30   As used herein, the term "enhanced" when used in reference to Vitaxin, a LM609 grafted antibody or a functional fragment thereof is intended to mean that a functional characteristic of the antibody has been

17

altered or augmented compared to a reference antibody so
that the antibody exhibits a desirable property or
activity. An antibody exhibiting enhanced activity can
exhibit, for example, higher affinity or lower affinity
5   binding, or increased or decreased association or
dissociation rates compared to a reference antibody. An
antibody exhibiting enhanced activity can also exhibit
increased stability such as increased half-life in a
particular organism. For example, if higher affinity
10   binding is desired, mutations can be introduced into
framework or CDR amino acid residues and the resulting
antibody variants screened for higher affinity binding to
$\alpha_v\beta_3$ relative to a reference antibody such as the LM609
grafted parent antibody.

15   The invention provides a nucleic acid encoding
a heavy chain polypeptide for Vitaxin or a functional
fragment thereof. Also provided is a nucleic acid
encoding a light chain polypeptide for Vitaxin or a
functional fragment thereof. The nucleic acids consist
20   of substantially the same heavy or light chain variable
region nucleotide sequences as those shown in Figure 1A
and 1B (SEQ ID NOS:1 and 3, respectively) or a fragment
thereof.

          Vitaxin, including functional fragments
25   thereof, is a non-mouse antibody which exhibits
substantially the same binding activity, binding
specificity and inhibitory activity as LM609. The
Vitaxin Fv Fragment was produced by functionally
replacing CDRs within human heavy and light chain
30   variable region polypeptides with the CDRs derived from
LM609. Functional replacement of the CDRs was performed
by recombinant methods known to those skilled in the art.
Such methods are commonly referred to as CDR grafting and

18

are the subject matter of U.S. Patent No. 5,225,539.
Such methods can also be found described in "Protein
Engineering of Antibody Molecules for Prophylactic and
Therapeutic Applications in Man," Clark, M. (ed.),
5   Nottingham, England: Academic Titles (1993).

        Briefly, LM609 nucleic acid fragments having
substantially the same nucleotide and encoding
substantially the same amino acid sequence of each of the
heavy and light chain CDRs were synthesized and
10   substituted into each of the respective human chain
encoding nucleic acids. To maintain functionality of the
newly derived Vitaxin antibody, modifications were
performed within the non-CDR framework region. These
individual changes were made by generating a population
15   of CDR grafted heavy and light chain variable regions
wherein all possible changes outside of the CDRs were
represented and then selecting the appropriate antibody
by screening the population for binding activity. This
screen resulted in the selection of the Vitaxin antibody
20   described herein.

        The nucleotide sequences of the Vitaxin heavy
and light chain variable regions are shown in Figures 1A
and 1B, respectively. These sequences correspond
substantially to those that encode the heavy and light
25   chain variable region polypeptides of Vitaxin. These
Vitaxin nucleic acids are intended to include both the
sense and anti-sense strands of the Vitaxin encoding
sequences. Single- and double-stranded nucleic acids are
similarly included as well as non-coding portions of the
30   nucleic acid such as introns, 5'- and 3'-untranslated
regions and regulatory sequences of the gene for example.

19

As shown in Figure 1A, the Vitaxin heavy chain variable region polypeptide is encoded by a nucleic acid of about 351 nucleotides in length which begins at the amino terminal Gln1 residue of the variable region

5   through to Ser117. This Vitaxin heavy chain variable region encoding nucleic acid is joined to a human IgG1 constant region to yield a coding region of 1431 nucleotides which encodes a heavy chain polypeptide of 477 total amino acids. Shown in Figure 1B is the Vitaxin

10 light chain variable region polypeptide which is encoded by a nucleic acid of about 321 nucleotides in length beginning at the amino terminal Glu1 residue of the variable region through to Lys107. This Vitaxin light chain variable region nucleic acid is joined to a human

15 kappa construct region to yield a coding region of 642 nucleotides which code for a light chain polypeptide of 214 total amino acids.

Minor modification of these nucleotide sequences are intended to be included as heavy and light

20 chain Vitaxin encoding nucleic acids and their functional fragments. Such minor modifications include, for example, those which do not change the encoded amino acid sequence due to the degeneracy of the genetic code as well as those which result in only a conservative

25 substitution of the encoded amino acid sequence. Conservative substitutions of encoded amino acids include, for example, amino acids which belong within the following groups: (1) non-polar amino acids (Gly, Ala, Val, Leu, and Ile); (2) polar neutral amino acids (Cys,

30 Met, Ser, Thr, Asn, and Gln); (3) polar acidic amino acids (Asp and Glu); (4) polar basic amino acids (Lys, Arg and His); and (5) aromatic amino acids (Phe, Trp, Tyr, and His). Other minor modifications are included within the nucleic acids encoding Vitaxin heavy and light

20

chain polypeptides so long as the nucleic acid or encoded
polypeptides retain some or all of their function as
described herein.

Thus, the invention also provides a nucleic
5   acid encoding a Vitaxin heavy chain or functional
fragment thereof wherein the nucleic acid encodes
substantially the same heavy chain variable region amino
acid sequence of Vitaxin as that shown in Figure 1A (SEQ
ID NO:2) or a fragment thereof. Similarly, the invention
10 also provides a nucleic acid encoding a Vitaxin light
chain or functional fragment thereof wherein the nucleic
acid encodes substantially the same light chain variable
region amino acid sequence of Vitaxin as that shown in
Figure 1B (SEQ ID NO:4) or a fragment thereof.
15

In addition to conservative substitutions of
amino acids, minor modifications of the Vitaxin encoding
nucleotide sequences which allow for the functional
replacement of amino acids are also intended to be
20 included within the definition of the term. The
substitution of functionally equivalent amino acids
encoded by the Vitaxin nucleotide sequences is routine
and can be accomplished by methods known to those skilled
in the art. Briefly, the substitution of functionally
25 equivalent amino acids can be made by identifying the
amino acids which are desired to be changed,
incorporating the changes into the encoding nucleic acid
and then determining the function of the recombinantly
expressed and modified Vitaxin polypeptide or
30 polypeptides. Rapid methods for making and screening
multiple simultaneous changes are well known within the
art and can be used to produce a library of encoding
nucleic acids which contain all possible or all desired
changes and then expressing and screening the library for

21

Vitaxin polypeptides which retain function. Such methods
include, for example, codon based mutagenesis, random
oligonucleotide synthesis and partially degenerate
oligonucleotide synthesis.

5   Codon based mutagenesis is the subject matter
of U.S. Patent Nos. 5,264,563 and 5,523,388 and is
advantageous for the above procedures since it allows for
the production of essentially any and all desired
frequencies of encoded amino acid residues at any and all
10 particular codon positions within an oligonucleotide.
Such desired frequencies include, for example, the truly
random incorporation of all twenty amino acids or a
specified subset thereof as well as the incorporation of
a predetermined bias of one or more particular amino
15 acids so as to incorporate a higher or lower frequency of
the biased residues compared to other incorporated amino
acid residues. Random oligonucleotide synthesis and
partially degenerate oligonucleotide synthesis can
similarly be used for producing and screening for
20 functionally equivalent amino acid changes. However, due
to the degeneracy of the genetic code, such methods will
incorporate redundancies at a desired amino acid
position. Random oligonucleotide synthesis is the
coupling of all four nucleotides at each nucleotide
25 position within a codon whereas partially degenerate
oligonucleotide synthesis is the coupling of equal
portions of all four nucleotides at the first two
nucleotide positions, for example, and equal portions of
two nucleotides at the third position. Both of these
30 latter synthesis methods can be found described in, for
example, Cwirla et al., Proc. Natl. Acad. Sci. USA
87:6378-6382, (1990) and Devlin et al., Science 249:404-
406, (1990).

22

Identification of amino acids to be changed can
be accomplished by those skilled in the art using current
information available regarding the structure and
function of antibodies as well as available and current
5   information encompassing methods for CDR grafting
procedures. For example, CDRs can be identified within
the donor antibody by any or all of the criteria
specified in Kabat et al., *supra*, Chothia et al., *supra*,
and/or MacCallum et al., *supra*, and any or all
10   non-identical amino acid residues falling outside of
these CDR sequences can be changed to functionally
equivalent amino acids. Using the above described
methods known within the art, any or all of the
non-identical amino acids can be changed either alone or
15   in combination with amino acids at different positions to
incorporate the desired number of amino acid
substitutions at each of the desired positions. The
Vitaxin polypeptides containing the desired substituted
amino acids are then produced and screened for retention
20   or augmentation of function compared to the unsubstituted
Vitaxin polypeptides. Production of the substituted
Vitaxin polypeptides can be accomplished by, for example,
recombinant expression using methods known to those
skilled in the art. Those Vitaxin polypeptides which
25   exhibit retention or augmentation of function compared to
unsubstituted Vitaxin are considered to contain minor
modifications of the encoding nucleotide sequence which
result in the functional replacement of one or more amino
acids.

30   The functional replacement of amino acids is
beneficial when producing grafted antibodies having human
framework sequences since it allows for the rapid
identification of equivalent amino acid residues without
the need for structural information or the laborious

23

procedures necessary to assess and identify which amino
acid residues should be considered for substitution in
order to successfully transfer binding function from the
donor. Moreover, it eliminates the actual step-wise
5   procedures to change and test the amino acids identified
for substitution. Essentially, using the functional
replacement approach described above, all non-identical
amino acid residues between the donor and the human
framework can be identified and substituted with any or
10   all other possible amino acid residues at each
non-identical position to produce a population of
substituted polypeptides containing all possible or all
desired permutations and combinations. The population of
substituted polypeptides can then be screened for those
15   substituted polypeptides which retain function. Using
the codon based mutagenesis procedures described above,
the generation of a library of substituted amino acid
residues and the screening of functionally replaced
residues has been used for the rapid production of
20   grafted therapeutic antibodies as well as for the rapid
alteration of antibody affinity. Such procedures are
exemplified in, for example, Rosok et al., J. Biol. Chem.
271:22611-22618 (1996) and in Glaser et al., J. Immunol.
149:3903-3913 (1992), respectively.

25   In addition to framework residues, amino acids
in one or more CDRs can be functionally replaced to allow
identification of a modified LM609 grafted antibody
having enhanced activity. Using the methods described
above for framework residues, amino acid substitutions
30   can similarly be introduced into one or more CDRs in an
LM609 grafted antibody. The modified LM609 grafted
antibody can be tested for binding activity to determine
whether $\alpha_v\beta_3$ binding activity is maintained. The modified
LM609 grafted antibody can be further tested to determine

24

if activity has been enhanced. Functional replacement of amino acid residues in one or more CDRs therefore allows the identification of an enhanced LM609 grafted antibody having a desirable property such as enhanced activity.

5   To generate modified LM609 grafted antibodies and select those with enhanced activity, several approaches can be employed in the selection of the number of residues within a CDR to mutate as well as the number of CDRs within a LM609 grafted antibody to modify. The

10   choice of selection criteria for mutagenesis of CDRs will depend on the need and desired application of the enhanced antibody. For example, one or a few amino acid positions within a single CDR can be modified to contain selected amino acids at that position. Alternatively,

15   the targeted amino acid positions can be modified to contain all possible amino acids at that position. The resultant population of modified antibodies can then be screened for enhanced activity.

     The construction of modified LM609 grafted

20   antibody populations can also be made where all amino acids positions within a CDR have been mutated to contain all possible amino acids and where amino acid positions within multiple CDRs have been modified to contain variant amino acid residues. In this way, populations

25   can be constructed which range from 2 to $>10^7$ unique members. The larger the population, the more efficient will be the selection of an enhanced LM609 grafted antibody since there will be a larger number of different antibodies within the population. However, a small

30   population of modified LM609 grafted antibodies can be made and successfully used for the selection of enhanced LM609 grafted antibodies. The size of the population of modified LM609 grafted antibodies will depend on the need

25

of a particular application and can be determined by one
skilled in the art.

The generation of modified LM609 grafted
antibodies can be achieved by introducing amino acid
5   substitutions into one or more CDRs of an LM609 grafted
antibody. For example, single amino acid substitutions
can be systematically introduced into a CDR by changing a
given amino acid in the CDR to any or all amino acids.
Amino acid substitutions can also be introduced into all
10   amino acid positions in one or more of the CDRs or in all
of the CDRs, generating a population of modified LM609
grafted antibody variants. This population of modified
LM609 grafted antibody variants having single amino acid
substitutions can be screened to identify those variants
15   that maintain $\alpha_v\beta_3$ binding activity. The variants having
$\alpha_v\beta_3$ binding activity can be further characterized to
identify those variants having enhanced activity. Such a
systematic approach to introducing single amino acid
substitutions and generating a population of LM609
20   grafted antibody variants to screen for enhanced LM609
grafted antibodies having high affinity binding to $\alpha_v\beta_3$ is
described in Example VI.

In addition to generating a population of
modified LM609 grafted antibody variants, a particular
25   CDR or a particular amino acid in a CDR can be selected
to introduce one or more amino acid substitutions. For
example, sequence homology or a structural model can be
used to identify particular amino acid positions to
introduce amino acid substitutions. In this example,
30   only one or a few modified LM609 grafted antibody
variants are generated and screened for binding activity
to $\alpha_v\beta_3$. One of skill in the art will know or can
determine whether it is desirable to generate a large

26

population of modified LM609 grafted antibody variants or
to generate a limited number of modified LM609 grafted
antibody variants to screen and identify an enhanced
LM609 grafted antibody having enhanced activity.

5   In addition to identifying enhanced LM609
grafted antibodies by generating a population of modified
LM609 grafted antibodies having single amino acid
substitutions in a CDR and screening for enhanced
activity, enhanced LM609 grafted antibody variants can
10   also be generated by combining two or more mutations,
each known to independently result in enhanced activity,
into a single antibody. When there are more than two
mutations, an efficient way to identify combinations of
mutations which further augment activity is to construct
15   all possible combinations and permutations and then
select for those with enhanced activity. For example,
two single mutations in one or more CDRs can be combined
to generate a new modified LM609 grafted antibody having
two CDR mutations and screened to determine if the $\alpha_v\beta_3$
20   binding activity is increased over that of the single
mutants. Similarly, three mutations can be combined and
the resulting modified LM609 grafted antibody screened
for enhanced binding activity. Using such an approach of
combining CDR mutations, a new population of modified
25   LM609 grafted antibody variants can be generated by
incorporating all combinations of the single CDR
mutations resulting in enhanced activity into new
modified LM609 grafted antibody variants and screening to
obtain an optimized enhanced LM609 grafted antibody.

30   An iterative, step-wise approach to identifying
an enhanced LM609 grafted antibody is advantageous in
that it allows the identification of an antibody having
optimal binding activity without the need to generate and

27

screen a large number of modified LM609 grafted antibody variants. For example, using the approach described in Examples VI and VII in which single mutants were identified and combined into a new population of LM609

5   grafted antibody variants, enhanced LM609 grafted antibodies having higher affinity were identified by generating 2592 unique variants. In contrast, complete randomization of a single eight amino acid residue CDR would require >10^{10} unique variants. Therefore, such an

10 iterative approach allows identification of enhanced LM609 grafted antibodies having enhanced activity such as high affinity binding by generating a relatively small number of unique modified LM609 grafted antibody variants and screening and identifying those enhanced LM609

15 grafted antibody variants exhibiting high affinity binding.

An iterative, step-wise approach to identifying enhanced LM609 variants can also be performed using additional steps. Instead of generating all combinations

20 of single amino acid mutations, the single amino acid mutations can be combined in pairs to generate all combinations of double mutants and screened for activity. Those double mutants having enhanced activity can be combined with any or all single mutants to generate

25 triple mutants that are screened for enhanced binding activity. Each iterative round of generating modified LM609 grafted antibody variants can incorporate additional single mutations, and the resulting modified LM609 grafted antibodies can be screened for enhanced

30 activity. The step-wise generation of LM609 grafted antibody variants can thus be used to identify an optimized LM609 grafted antibody. Additionally, such an iterative approach also allows for the identification of

28

numerous enhanced antibodies which exhibit a range of
different, enhanced binding activities.

An optimized LM609 grafted antibody can also be
referred to as an LM609-like grafted antibody or an
5   $\alpha_v\beta_3$-specific grafted antibody and is recognizable because
the antibody or functional fragments thereof retains the
functional characteristics of LM609. For example,
enhanced LM609 grafted antibody variants, which have a
single amino acid substitution and have enhanced
10 activity, can be identified and correlated with a
specific amino acid substitution. These amino acid
substitutions can be combined to generate a new modified
LM609 grafted antibody that is tested for activity. Such
a combination of advantageous CDR amino acid
15 substitutions can result in an optimized LM609 grafted
antibody with multiple CDRs having at least one amino
acid substitution or a single CDR having multiple amino
acid substitutions, where the modified LM609 grafted
antibody has enhanced activity.

20   Enhanced LM609 grafted antibodies, particularly
those optimized by functional replacement of amino acid
residues in the CDRs, have desirable enhanced properties
such as increased affinity. For example, an optimized
LM609 grafted antibody having increased affinity will
25 have higher affinity than the parent antibody used for
introducing functional replacement of amino acids.
Higher affinity is determined relative to a reference
antibody having a similar structure. For example, if the
optimized LM609 grafted antibody is an intact antibody
30 containing two heavy chains and two light chains, then
higher affinity is determined relative to the intact
parent LM609 grafted antibody. Similarly, if the
optimized LM609 grafted antibody is an Fab, then higher

29

affinity is determined relative to the Fab of the parent LM609 grafted antibody.

Although it is not necessary to proceed through multiple optimization steps to obtain a high affinity LM609 grafted antibody, in general, the increase in affinity can correlate with the number of modifications within and between CDRs as well as with the number of optimization steps. Therefore, LM609 grafted antibodies will exhibit a variety of ranges. For example, LM609 grafted antibodies having enhanced affinity will have up to about 2-fold higher affinity or greater, generally greater than about 2- to 5-fold higher affinity such as greater than about 4- to 5-fold higher affinity or about 5- to 10-fold higher affinity than the reference antibody. Particularly, a LM609 grafted antibody having enhanced affinity will have greater than about 10- to 50-fold higher affinity, greater than about 50-fold higher affinity, or greater than about 100-fold higher affinity than the reference antibody.

As described above, functional replacement of CDR amino acid residues can be used to identify LM609 grafted antibodies exhibiting higher affinity than a parent LM609 grafted antibody. Methods discussed above or below for introducing minor modifications into Vitaxin or LM609 grafted antibody encoding nucleotide sequences can similarly be used to generate a library of modified LM609 grafted antibody variants, including methods such as codon based mutagenesis, random oligonucleotide synthesis and partially degenerate oligonucleotide synthesis. For example, codon based mutagenesis has been used to generate such a library of modified LM609 grafted antibody variants having single amino acid substitutions (see Example VI).

30

After generating a library of modified LM609 grafted antibody variants, the variants can be expressed and screened for binding activity to $\alpha_v\beta_3$. Methods well known to those skilled in the art related to determining antibody-antigen interactions are used to screen for modified LM609 grafted antibodies exhibiting binding activity to $\alpha_v\beta_3$ (Harlow and Lane, *supra*). For example, an ELISA method has been used to screen a library of modified LM609 grafted antibody variants to identify those variants that maintained $\alpha_v\beta_3$ binding activity (see Example VI). Only those modified LM609 grafted antibodies that maintain $\alpha_v\beta_3$ binding activity are considered for further characterization.

Modified LM609 grafted antibodies having $\alpha_v\beta_3$ binding activity can be further characterized to determine which modified LM609 grafted antibody has enhanced activity. The type of assay used to assess enhanced activity depends on the particular desired characteristic. For example, if altered binding activity is desired, then binding assays that allow determination of binding affinity are used. Such assays include binding assays, competition binding assays and surface plasmon resonance as described in Example VI.

Introduction of single amino acid substitutions into CDRs of LM609 grafted antibodies can be used to generate a library of modified LM609 grafted antibodies and screen for binding activity to $\alpha_v\beta_3$. Those modified LM609 grafted antibodies exhibiting binding activity to $\alpha_v\beta_3$ can then be further characterized to identify enhanced LM609 grafted antibodies exhibiting enhanced activity such as higher binding affinity. For example, using such an approach, a number of enhanced LM609 grafted antibodies having single amino acid substitutions

31

were generated using the heavy chain variable region
shown in Figure 1a (SEQ ID NO:2) and the light chain
variable region shown in Figure 7 (SEQ ID NO:32), and
LM609 grafted antibodies were identified displaying 2 to
5   13-fold improved affinity over the parent LM609 grafted
antibody (see Example VI).

        Following identification of enhanced LM609
grafted antibodies having a single amino acid
substitution, the amino acid mutations can be combined to
10 further enhance activity. Methods discussed above for
introducing single amino acid substitutions into CDRs can
similarly be applied to combine amino acid substitutions.
For example, a combinatorial library of amino acid
mutations that resulted in enhanced $\alpha_V\beta_3$ binding affinity
15 was generated using degenerate oligonucleotides and two
site hybridization mutagenesis as described in Example
VII. Enhanced LM609 grafted antibodies containing
multiple CDR amino acid substitutions were generated
using the heavy chain variable region shown in Figure 1a
20 (SEQ ID NO:2) and the light chain variable region shown
in Figure 7 (SEQ ID NO:32), and LM609 grafted antibodies
were identified having 20-fold higher affinity to greater
than 90-fold higher affinity than the parent LM609
grafted antibody.

25   In addition to combining CDR amino acid
substitutions to generate an enhanced or optimized LM609
grafted antibody, CDR amino acid substitutions can also
be combined with framework mutations that contribute
desirable properties to a LM609 grafted antibody. Thus,
30 mutations in CDR or framework regions that enhance
activity can be combined to further optimize LM609
grafted antibodies.

32

The invention further provides fragments of
Vitaxin heavy and light chain encoding nucleic acids
wherein such fragments consist substantially of the same
nucleotide or amino acid sequence as the variable region
5   of Vitaxin heavy or light chain polypeptides. The
variable region of the Vitaxin heavy chain polypeptide
consists essentially of nucleotides 1-351 and of amino
acid residues Gln1 to Ser117 of Figure 1A (SEQ ID NOS:1
and 2, respectively). The variable region of the Vitaxin
10 light chain polypeptide consists essentially of
nucleotides 1-321 and of amino acid residues Glu1 to
Lys107 of Figure 1B (SEQ ID NOS:3 and 4, respectively).
The termini of such variable region encoding nucleic
acids is not critical so long as the intended purpose and
15 function remains the same.

Fragments additional to the variable region
nucleic acid fragments are provided as well. Such
fragments include, for example, nucleic acids consisting
substantially of the same nucleotide sequence as a CDR of
20 a Vitaxin heavy or light chain polypeptide. Sequences
corresponding to the Vitaxin CDRs include, for example,
those regions defined by Kabat et al., *supra*, and/or
those regions defined by Chothia et al., *supra*, as well
as those defined by MacCallum et al., *supra*. The Vitaxin
25 CDR fragments for each of the above definitions
correspond to the nucleotides set forth below in Table 2.
The nucleotide sequence numbering is taken from the
primary sequence shown in Figures 1A and 1B (SEQ ID NOS:1
and 3) and conforms to the definitions previously set
30 forth in Table 1.

33

Table 2: Vitaxin CDR Nucleotide Residues

	Kabat	Chothia	MacCallum
V$_H$ CDR1	91-105	76-96	88-105
V$_H$ CDR2	148-198	157-168	139-177
V$_H$ CDR3	295-318	298-315	289-315
V$_L$ CDR1	70-102	76-96	88-108
V$_L$ CDR2	148-168	148-156	136-165
V$_L$ CDR3	265-291	271-288	265-288

Similarly, the Vitaxin CDR fragments for each
of the above definitions correspond to the amino acid
residues set forth below in Table 3. The amino acid
residue number is taken from the primary sequence shown
in Figures 1A and 1B (SEQ ID NOS:2 and 4) and conforms to
the definitions previously set forth in Table 1.

Table 3: Vitaxin CDR Amino Acid Residues

	Kabat	Chothia	MacCallum
V$_H$ CDR1	Ser31-Ser35	Gly26-Tyr32	Ser30-Ser35
V$_H$ CDR2	Lys50-Gly66	Ser53-Gly56	Trp47-Tyr59
V$_H$ CDR3	His99-Tyr106	Asn100-Ala105	Ala97-Ala105
V$_L$ CDR1	Gln24-His34	Ser26-His32	Ser30-Tyr36
V$_L$ CDR2	Tyr50-Ser56	Tyr50-Ser52	Leu46-Ile55
V$_L$ CDR3	Gln89-Thr97	Ser91-His96	Gln89-His96

Thus, the invention also provides nucleic acid
fragments encoding substantially the same amino acid
sequence as a CDR of a Vitaxin heavy or light chain
polypeptide.

Nucleic acids encoding Vitaxin heavy and light
chain polypeptides and fragments thereof are useful for a
variety of diagnostic and therapeutic purposes. For

34

example, the Vitaxin nucleic acids can be used to produce
Vitaxin antibodies and functional fragments thereof
having binding specificity and inhibitory activity
against the integrin $\alpha_v\beta_3$. The antibody and functional
5   fragments thereof can be used for the diagnosis or
therapeutic treatment of $\alpha_v\beta_3$-mediated disease. Vitaxin
and functional fragments thereof can be used, for
example, to inhibit binding activity or other functional
activities of $\alpha_v\beta_3$ that are necessary for progression of
10   an $\alpha_v\beta_3$-mediated disease. Other functional activities
necessary for progression of $\alpha_v\beta_3$-mediated disease
include, for example, the activation of $\alpha_v\beta_3$, $\alpha_v\beta_3$-mediated
signal transduction and the $\alpha_v\beta_3$-mediated prevention of
apoptosis. Advantageously, however, Vitaxin comprises
15   non-mouse framework amino acid sequences and as such is
less antigenic in regard to the induction of a host
immune response. The Vitaxin nucleic acids of the
inventions can also be used to model functional
equivalents of the encoded heavy and light chain
20   polypeptides.

Thus, the invention provides Vitaxin heavy
chain and Vitaxin light chain polypeptides or functional
fragments thereof. The Vitaxin heavy chain polypeptide
exhibits substantially the same amino acid sequence as
25   that shown in Figure 1A (SEQ ID NO:2) or functional
fragment thereof whereas the Vitaxin light chain
polypeptide exhibits substantially the same amino acid
sequence as that shown in Figure 1B (SEQ ID NO:4) or
functional fragment thereof. Also provided is a Vitaxin
30   antibody or functional fragment thereof. The antibody is
generated from the above heavy and light chain
polypeptides or functional fragments thereof and exhibits
selective binding affinity to $\alpha_v\beta_3$.

35

The invention provides a nucleic acid encoding
a heavy chain polypeptide for a LM609 grafted antibody.
Also provided is a nucleic acid encoding a light chain
polypeptide for a LM609 grafted antibody. The nucleic
5   acids consist of substantially the same heavy chain
variable region nucleotide sequence as that shown in
Figure 1A (SEQ ID NO:1) and substantially the same light
chain variable region nucleotide sequence as that shown
in Figure 7 (SEQ ID NO:31) or a fragment thereof.

10   LM609 grafted antibodies, including functional
fragments thereof, are non-mouse antibodies which exhibit
substantially the same binding activity, binding
specificity and inhibitory activity as LM609. The LM609
grafted antibody Fv fragments described herein are
15   produced by functionally replacing the CDRs as defined by
Kabat et al. , hereinafter referred to as "Kabat CDRs,"
within human heavy and light chain variable region
polypeptides with the Kabat CDRs derived from LM609.
Functional replacement of the Kabat CDRs is performed by
20   the CDR grafting methods previously described and which
is the subject matter of U.S. Patent No. 5,225,539,
supra. Substitution of amino acid residues outside of
the Kabat CDRs can additionally be performed to maintain
or augment beneficial binding properties so long as such
25   amino acid substitutions do not correspond to a donor
amino acid at that particular position. Such
substitutions allow for the modulation of binding
properties without imparting any mouse sequence
characteristics onto the antibody outside of the Kabat
30   CDRs. Although the production of such antibodies is
described herein with reference to LM609 grafted
antibodies, the substitution of such non-donor amino
acids outside of the Kabat CDRs can be utilized for the
production of essentially any grafted antibody. The

36

production of LM609 grafted antibodies is described
further below in Example V.

The nucleotide sequences of the LM609 grafted
antibody heavy and light chain variable regions are shown

5   in Figures 1A and 7, respectively. These sequences
correspond substantially to those that encode the heavy
and light chain variable region polypeptides of a LM609
grafted antibody. These nucleic acids are intended to
include both the sense and anti-sense strands of the

10   LM609 grafted antibody encoding sequences. Single- and
double-stranded nucleic acids are similarly included as
well as non-coding portions of the nucleic acid such as
introns, 5'- and 3'-untranslated regions and regulatory
sequences of the gene for example.

15   The nucleotide and amino acid residue
boundaries for a LM609 grafted antibody are identical to
those previously described for Vitaxin. For example, a
LM609 grafted antibody heavy chain variable region
polypeptide is encoded by a nucleic acid of about 351

20   nucleotides in length which begins at the amino terminal
Gln1 residue of the variable region through to Ser117
(Figure 1A, SEQ ID NOS:1 and 2, respectively). The LM609
grafted antibody light chain variable region polypeptide
is encoded by a nucleic acid of about 321 nucleotides in

25   length beginning at the amino terminal Glu1 residue of
the variable region through to Lys107 (Figure 7, SEQ ID
NOS:31 and 32, respectively). As with Vitaxin, minor
modification of these nucleotide sequences are intended
to be included as heavy and light chain variable region

30   encoding nucleic acids and their functional fragments.

Thus, the invention also provides a nucleic
acid encoding a LM609 grafted antibody heavy chain

37

wherein the nucleic acid encodes substantially the same
heavy chain variable region amino acid sequence as that
shown in Figure 1A (SEQ ID NO:2) or fragment thereof.
Similarly, the invention also provides a nucleic acid

5   encoding a LM609 grafted antibody light chain wherein the
nucleic acid encodes substantially the same light chain
variable region amino acid sequence as that shown in
Figure 7 (SEQ ID NO:32) or fragment thereof.

         In addition to conservative substitutions of
10   amino acids, minor modifications of the LM609 grafted
antibody encoding nucleotide sequences which allow for
the functional replacement of amino acids are also
intended to be included within the definition of the
term. Identification of amino acids to be changed can be
15   accomplished by those skilled in the art using current
information available regarding the structure and
function of antibodies as well as available and current
information encompassing methods for CDR grafting
procedures. The substitution of functionally equivalent
20   amino acids encoded by the LM609 grafted antibody
nucleotide sequences is routine and can be accomplished
by methods known to those skilled in the art. As
described previously, such methods include, for example,
codon based mutagenesis, random oligonucleotide synthesis
25   and partially degenerate oligonucleotide synthesis and
are beneficial when producing grafted antibodies since
they allow for the rapid identification of equivalent
amino acid residues without the need for structural
information.

30   The invention further provides fragments of
LM609 grafted antibody heavy and light chain encoding
nucleic acids wherein such fragments consist
substantially of the same nucleotide or amino acid

38

sequence as the variable region of a LM609 grafted
antibody heavy or light chain polypeptide. As with
Vitaxin, the termini of such variable region encoding
nucleic acids is not critical so long as the intended
5   purpose and function remains the same.

Fragments additional to the variable region
nucleic acid fragments are provided as well and include,
for example, nucleic acids consisting substantially of
the same nucleotide sequence as a CDR of a LM609 grafted
10 antibody heavy or light chain polypeptide. As with
Vitaxin, sequences corresponding to the LM609 grafted
antibody CDRs include, for example, those regions defined
by Kabat et al., *supra*, Chothia et al., *supra*, as well as
those defined by MacCallum et al., *supra*. The LM609
15 grafted antibody CDR regions will be similar to those
described previously for Vitaxin. Moreover, such regions
are well known and can be determined by those skilled in
the art given the LM609 sequences and teachings provided
herein. Thus, the invention also provides nucleic acid
20 fragments encoding substantially the same amino acid
sequence as a CDR of a LM609 grafted antibody heavy or
light chain polypeptide.

As with Vitaxin, nucleic acids encoding LM609
grafted antibody heavy and light chain polypeptides and
25 fragments thereof are useful for a variety of diagnostic
and therapeutic purposes. For example, LM609 grafted
antibody encoding nucleic acids can be used to produce
recombinant antibodies and functional fragments thereof
having binding specificity and inhibitory activity
30 against the integrin $\alpha_v\beta_3$. The antibody and functional
fragments thereof can be used for the diagnosis or
therapeutic treatment of $\alpha_v\beta_3$-mediated disease. Such
diseases and methods of use for anti-$\alpha_v\beta_3$ antibodies have

39

been described previously in reference to Vitaxin and are
equally applicable to the LM609 grafted antibodies
described herein.

   Thus, the invention provides LM609 grafted
5 antibody heavy chain and Vitaxin light chain polypeptides
or functional fragments thereof. The LM609 grafted
antibody heavy chain polypeptide exhibits substantially
the same amino acid sequence as that shown in Figure 1A
(SEQ ID NO:2) or functional fragment thereof whereas the
10 LM609 grafted antibody light chain polypeptide exhibits
substantially the same amino acid sequence as that shown
in Figure 7 (SEQ ID NO:32). Also provided is a LM609
grafted antibody or functional fragment thereof. The
antibody is generated from the above heavy and light
15 chain polypeptides or functional fragments thereof and
exhibits selective binding affinity to $\alpha_v\beta_3$.

   The invention provides an enhanced LM609
grafted antibody exhibiting selective binding affinity to
$\alpha_v\beta_3$. The enhanced LM609 grafted antibody contains at
20 least one amino acid substitution in one or more CDRs of
a LM609 grafted heavy chain variable region polypeptide
or a LM609 grafted light chain variable region
polypeptide, wherein the $\alpha_v\beta_3$ binding affinity of the
enhanced LM609 grafted antibody is maintained or
25 enhanced.

   To identify enhanced LM609 grafted antibodies,
a library of modified LM609 grafted antibodies was
generated as described above and in Example VI.
Initially, LM609 CDRs were identified and selected to
30 introduce single amino acid substitutions. Utilizing the
numbering system of Kabat et al., supra, the CDR residues
selected for mutagenesis were V_H CDR1 Gly-Phe-Thr-Phe-Ser-

40

Ser-Tyr-Asp-Met-Ser (SEQ ID NO:34) (Gly^{26}-Ser^{35}); V_H CDR2
Trp-Val-Ala-Lys-Val-Ser-Ser-Gly-Gly-Gly (SEQ ID NO:36)
and Ser-Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Gln-Gly (SEQ ID
NO:38) (Trp^{47}-Gly^{65}); V_H CDR3 Ala-Arg-His-Asn-Tyr-Gly-Ser-

5   Phe-Ala-Tyr (SEQ ID NO:40) (Ala^{93}-Tyr^{102}); V_L CDR1 Gln-Ala-
Ser-Gln-Ser-Ile-Ser-Asn-His-Leu-His-Trp-Tyr (SEQ ID
NO:42) (Gln^{24}-Tyr^{36}); V_L CDR2 Leu-Leu-Ile-Arg-Tyr-Arg-Ser-
Gln-Ser-Ile-Ser (SEQ ID NO:44) (Leu^{46}-Ser^{56}); and V_L CDR3
Gln-Gln-Ser-Gly-Ser-Trp-Pro-His-Thr (SEQ ID NO:46)

10 (Gln^{89}-Thr^{97}).

The nucleotide sequences encoding the CDR residues
selected for mutagenesis were V_H CDR1 GGA TTC ACC TTC AGT
AGC TAT GAC ATG TCT (SEQ ID NO:33); V_H CDR2 TGG GTC GCA AAA
GTT AGT AGT GGT GGT GGT (SEQ ID NO:35) and AGC ACC TAC

15 TAT TTA GAC ACT GTG CAG GGC (SEQ ID NO:37); V_H CDR3 GCA
AGA CAT AAC TAC GGC AGT TTT GCT TAC (SEQ ID NO:39); V_L
CDR1 CAG GCC AGC CAA AGT ATT AGC AAC CAC CTA CAC TGG TAT
(SEQ ID NO:41); V_L CDR2 CTT CTC ATC CGT TAT CGT TCC CAG
TCC ATC TCT (SEQ ID NO:43); and V_L CDR3 CAA CAG AGT GGC

20 AGC TGG CCT CAC ACG (SEQ ID NO:45).

Single amino acid substitutions can be
introduced into the CDRs of an LM609 grafted antibody to
generate a population of modified LM609 grafted
antibodies. For example, every amino acid in one or more

25 CDRs can be mutated to any or all amino acids to generate
a population of modified LM609 grafted antibodies and the
population screened for $\alpha_v\beta_3$ binding activity. Although
this population is generated by mutating amino acids in
CDRs, populations can also be constructed where changes

30 are made in the framework region residues or in both the
CDRs and the framework. Such mutations in the variable
regions can be made separately, in combination, or step-
wise. Thus, the invention also provides for an enhanced

41

LM609 grafted antibody, where the amino acid substitution is in the CDR or in the framework region.

The invention additionally provides an enhanced LM609 grafted antibody exhibiting enhanced binding
5 affinity. Enhanced LM609 grafted antibodies exhibiting enhanced binding affinity include those containing at least one of the following CDRs having single amino acid substitutions:
   a V_H CDR1 selected from the group consisting of Gly-Thr-
10 Thr-Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:48), Gly-Phe-Thr-Trp-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:50) and Gly-Phe-Thr-Phe-Leu-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:52);
   a V_H CDR2 selected from the group consisting of Trp-Val-Ala-Lys-Val-Lys-Ser-Gly-Gly-Gly (SEQ ID NO:54), Ser-Thr-
15 Tyr-Tyr-Pro-Asp-Thr-Val-Gln-Gly (SEQ ID NO:56) and Ser-Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Glu-Gly (SEQ ID NO:58);
   a V_H CDR3 selected from the group consisting of Ala-Arg-His-Asn-His-Gly-Ser-Phe-Ala-Tyr (SEQ ID NO:60), Ala-Arg-His-Asn-Tyr-Gly-Ser-Tyr-Ala-Tyr (SEQ ID NO:62), Ala-Arg-
20 His-Asn-Tyr-Gly-Ser-Phe-Asp-Tyr (SEQ ID NO:64), Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Tyr-Tyr (SEQ ID NO:66), Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:68), Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Thr (SEQ ID NO:70), Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Asp (SEQ ID NO:72), Ala-Arg-
25 His-Asn-Tyr-Gly-Ser-Phe-Ala-Glu (SEQ ID NO:74), Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Met (SEQ ID NO:76), Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Gly (SEQ ID NO:78) and Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Ala (SEQ ID NO:80);
   the V_L CDR1 Gln-Ala-Ser-Gln-Ser-Ile-Ser-Asn-Phe-Leu-His-
30 Trp-Tyr (SEQ ID NO:82); the V_L CDR2 Leu-Leu-Ile-Arg-Tyr-Ser-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:84); and
   a V_L CDR3 selected from the group consisting of Gln-Gln-Ser-Asn-Ser-Trp-Pro-His-Thr (SEQ ID NO:86), Gln-Gln-Ser-Thr-Ser-Trp-Pro-His-Thr (SEQ ID NO:88), Gln-Gln-Ser-Gly-

42

Ser-Trp-Pro-Leu-Thr (SEQ ID NO:90) and Gln-Gln-Ser-Gly-
Ser-Trp-Pro-Gln-Thr (SEQ ID NO:92).

The nucleotide sequences encoding the CDRs
having single amino acid substitutions were V_H CDR1 GGA

5   ACT ACC TTC AGT AGC TAT GAC ATG TCT (SEQ ID NO:47), GGA
TTC ACC TGG AGT AGC TAT GAC ATG TCT (SEQ ID NO:49), and
GGA TTC ACC TTC CTG AGC TAT GAC ATG TCT (SEQ ID NO:51); V_H
CDR2 TGG GTC GCA AAA GTT AAA AGT GGT GGT GGT (SEQ ID
NO:53), AGC ACC TAC TAT CCT GAC ACT GTG CAG GGC (SEQ ID

10 NO:55), and AGC ACC TAC TAT TTA GAC ACT GTG GAG GGC (SEQ
ID NO:57); V_H CDR3 GCA AGA CAT AAC CAT GGC AGT TTT GCT TAC
(SEQ ID NO:59), GCA AGA CAT AAC TAC GGC AGT TAT GCT TAC
(SEQ ID NO:61), GCA AGA CAT AAC TAC GGC AGT TTT GAT TAC
(SEQ ID NO:63), GCA AGA CAT AAC TAC GGC AGT TTT TAT TAC

15 (SEQ ID NO:65), GCA AGA CAT AAC TAC GGC AGT TTT GCT TCT
(SEQ ID NO:67), GCA AGA CAT AAC TAC GGC AGT TTT GCT ACT
(SEQ ID NO:69), GCA AGA CAT AAC TAC GGC AGT TTT GCT GAT
(SEQ ID NO:71), GCA AGA CAT AAC TAC GGC AGT TTT GCT GAG
(SEQ ID NO:73), GCA AGA CAT AAC TAC GGC AGT TTT GCT ATG

20 (SEQ ID NO:75), GCA AGA CAT AAC TAC GGC AGT TTT GCT GGG
(SEQ ID NO:77), and GCA AGA CAT AAC TAC GGC AGT TTT GCT
GCT (SEQ ID NO:79); V_L CDR1 CAG GCC AGC CAA AGT ATT AGC
AAC TTT CTA CAC TGG TAT (SEQ ID NO:81); V_L CDR2 CTT CTC
ATC CGT TAT TCT TCC AGT CCA TCT CCT (SEQ ID NO:83); and V_L

25 CDR3 CAA CAG AGT AAT AGC TGG CCT CAC ACG (SEQ ID NO:85),
CAA CAG AGT ACT AGC TGG CCT CAC ACG (SEQ ID NO:87), CAA
CAG AGT GGC AGC TGG CCT CTG ACG (SEQ ID NO:89) and CAA
CAG AGT GGC AGC TGG CCT CAG ACG (SEQ ID NO:91).

Enhanced LM609 grafted antibodies having CDRs

30 with single amino acid substitutions and higher affinity
binding than the parent LM609 grafted antibody can also
be identified, where the corresponding amino acid
mutations are combined to generate new modified LM609

43

grafted antibodies.   Identification is performed by
screening for $\alpha_v\beta_3$ binding activity.   In some
combinations, the LM609 grafted antibody will comprise at
least one CDR having two or more amino acid

5   substitutions.   The invention provides an enhanced LM609
grafted antibody containing at least one of the following
CDRs containing multiple amino acid substitutions: a V_H
CDR3 selected from the group consisting of Ala-Arg-His-
Asn-His-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:94); Ala-Arg-His-

10   Asn-His-Gly-Ser-Phe-Tyr-Ser (SEQ ID NO:96); Ala-Arg-His-
Asn-Tyr-Gly-Ser-Phe-Tyr-Glu (SEQ ID NO:98); and Ala-Arg-
His-Asn-Tyr-Gly-Ser-Phe-Tyr-Ser (SEQ ID NO:100).

The nucleotide sequences encoding the CDRS
having multiple amino acid substitutions were V_H CDR3 GCA

15   AGA CAT AAC CAT GGC AGT TTT GCT TCT (SEQ ID NO:93), GCA
AGA CAT AAC CAT GGC AGT TTT TAT TCT (SEQ ID NO:95), GCA
AGA CAT AAC TAC GGC AGT TTT TAT GAG (SEQ ID NO:97), and
GCA AGA CAT AAC TAC GGC AGT TTT TAT TCT (SEQ ID NO:99).

The invention also provides an enhanced LM609

20   grafted antibody exhibiting selective binding affinity to
$\alpha_v\beta_3$, wherein the enhanced LM609 grafted antibody contains
at least one amino acid substitution in two or more CDRs
of a LM609 grafted heavy chain variable region
polypeptide or a LM609 grafted light chain variable

25   region polypeptide.

An enhanced LM609 grafted antibody containing
at least one amino acid substitution in two or more CDRs
of a LM609 grafted heavy chain variable region
polypeptide or a LM609 grafted light chain variable

30   region polypeptide can include an LM609 grafted antibody
containing the combination of CDRs selected from the
group consisting of: the V_L CDR1 SEQ ID NO:57 and the V_H

44

CDR3 SEQ ID NO:50; the V_L CDR1 SEQ ID NO:57, the V_H CDR2
SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:50; the V_L CDR1 SEQ
ID NO:57, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID
NO:52; the V_L CDR1 SEQ ID NO:57, the V_H CDR2 SEQ ID NO:44

5   and the V_H CDR3 SEQ ID NO:51; the V_L CDR1 SEQ ID NO:57 and
the V_H CDR3 SEQ ID NO:52; the V_L CDR3 SEQ ID NO:59, the V_H
CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:50; the V_L
CDR3 SEQ ID NO:61 and V_H CDR3 SEQ ID NO:50; and the V_L
CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and V_H CDR3

10   SEQ ID NO:50.

In addition to enhanced LM609 grafted
antibodies containing two or more CDRs having single
amino acid substitutions, the invention also provides
enhanced LM609 grafted antibodies wherein at least one of

15   the CDRs has two or more amino acid substitutions.

Enhanced LM609 grafted antibodies having at
least one CDR with two or more amino acid substitutions
can include those containing the combination of CDRs
selected from the group consisting of: the V_L CDR1 SEQ ID

20   NO:57, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID
NO:63; the V_L CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44
and the V_H CDR3 SEQ ID NO:63; the V_L CDR3 SEQ ID NO:61,
the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:64; the
V_L CDR3 SEQ ID NO:61 and the V_H CDR3 SEQ ID NO:63; the V_L

25   CDR3 SEQ ID NO:61 and the V_H CDR3 SEQ ID NO:65; and the V_L
CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and the V_H
CDR3 SEQ ID NO:66.

The invention additionally provides a high
affinity LM609 grafted antibody exhibiting selective

30   binding affinity to $\alpha_v\beta_3$. The high affinity LM609 grafted
antibody contains at least one amino acid substitution in
one or more CDRs of a LM609 grafted heavy chain variable

region polypeptide or a LM609 grafted light chain
variable region polypeptide, wherein the $\alpha_v\beta_3$ binding
affinity of the high affinity LM609 grafted antibody is
enhanced.

5   High affinity antibodies can include those
containing the combination of CDRs selected from the
group consisting of: the V_L CDR1 SEQ ID NO:57 and the V_H
CDR3 SEQ ID NO:50; the V_L CDR1 SEQ ID NO:57, the V_H CDR2
SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:50; the V_L CDR1 SEQ
10   ID NO:57, the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID
NO:52; the V_L CDR1 SEQ ID NO:57, the V_H CDR2 SEQ ID NO:44
and the V_H CDR3 SEQ ID NO:51; the V_L CDR1 SEQ ID NO:57 and
the V_H CDR3 SEQ ID NO:52; the V_L CDR3 SEQ ID NO:59, the V_H
CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:50; the V_L
15   CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and the V_H
CDR3 SEQ ID NO:63; the V_L CDR3 SEQ ID NO:61 and V_H CDR3
SEQ ID NO:50; the V_L CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID
NO:44 and V_H CDR3 SEQ ID NO:50; the V_L CDR1 SEQ ID NO:57,
the V_H CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:63; the
20   V_L CDR3 SEQ ID NO:61, the V_H CDR2 SEQ ID NO:44 and the V_H
CDR3 SEQ ID NO:64; the V_L CDR3 SEQ ID NO:61 and the V_H
CDR3 SEQ ID NO:63; the V_L CDR3 SEQ ID NO:61 and the V_H
CDR3 SEQ ID NO:65; and the V_L CDR3 SEQ ID NO:61, the V_H
CDR2 SEQ ID NO:44 and the V_H CDR3 SEQ ID NO:66.

25   The invention additionally provides a nucleic
acid encoding an enhanced LM609 grafted antibody
exhibiting selective binding affinity to $\alpha_v\beta_3$. The
enhanced LM609 grafted antibody encoded by the nucleic
acid contains at least one amino acid substitution in one
30   or more CDRs of a LM609 grafted heavy chain variable
region polypeptide or a LM609 grafted light chain
variable region polypeptide, wherein the $\alpha_v\beta_3$ binding

46

affinity of the enhanced LM609 grafted antibody is
maintained or enhanced.

The invention further provides a nucleic acid
encoding a high affinity LM609 grafted antibody

5   exhibiting selective binding affinity to $\alpha_v\beta_3$. The high
affinity LM609 grafted antibody encoded by the nucleic
acid contains at least one amino acid substitution in one
or more CDRs of a LM609 grafted heavy chain variable
region polypeptide or a LM609 grafted light chain

10   variable region polypeptide, wherein the $\alpha_v\beta_3$ binding
affinity of the high affinity LM609 grafted antibody is
enhanced.

The invention provides a nucleic acid encoding
a heavy chain polypeptide for monoclonal antibody LM609

15   or functional fragment thereof. Also provided is a
nucleic acid encoding a light chain polypeptide for
monoclonal antibody LM609 or a functional fragment
thereof. The nucleic acids consist of substantially the
same heavy or light chain variable region nucleotide

20   sequences as that shown in Figure 2A and 2B (SEQ ID NOS:5
and 7, respectively) or a fragment thereof.

As described previously, monoclonal antibody
LM609 has been shown in the art to have binding activity
to the integrin $\alpha_v\beta_3$. Although specificity can in

25   principle be generated towards essentially any target,
LM609 is an integrin inhibitory antibody that exhibits
substantial specificity and inhibitory activity to a
single member within an integrin family. In this case,
LM609 exhibits substantial specificity and inhibitory

30   activity to the $\alpha_v\beta_3$ integrin within the β_3 family. The
amino acid or nucleotide sequence of monoclonal antibody

47

LM609 has never been previously isolated and
characterized.

The isolation and characterization of LM609
encoding nucleic acids was performed by techniques known
5   to those skilled in the art and which are described
further below in the Examples. Briefly, cDNA from
hybridoma LM609 was generated and used as the source for
which to isolate LM609 encoding nucleic acids. Isolation
was performed by first determining the N-terminal amino
10 acid sequence for each of the heavy and light chain
polypeptides and then amplifying by PCR the antibody
encoding sequences from the cDNA. The 5' primers were
reverse translated to correspond to the newly determined
N-terminal amino acid sequences whereas the 3' primers
15 corresponded to sequences substantially similar to
antibody constant region sequences. Amplification and
cloning of the products resulted in the isolation of the
nucleic acids encoding heavy and light chains of LM609.

The nucleotide sequences of the LM609 heavy and
20 light chain variable region sequences are shown in Figure
2A and 2B, respectively. These sequences correspond
substantially to those that encode the variable region
heavy and light chain polypeptides of LM609. As with the
Vitaxin nucleic acids, these LM609 nucleic acids are
25 intended to include both sense and anti-sense strands of
the LM609 encoding sequences. Single- and double-
stranded nucleic acids are also include as well as
non-coding portions of the nucleic acid such as introns,
5'- and 3'-untranslated regions and regulatory sequences
30 of the gene for example.

As shown in Figure 2A, the LM609 heavy chain
variable region polypeptide is encoded by a nucleic acid

48

of about 351 nucleotides in length which begins at the
amino terminal Glu1 residue of the variable region
through to Ala 117.   The murine LM609 antibody heavy
chain has an IgG2a constant region.   Shown in Figure 2B

5   is the LM609 light chain variable region polypeptide
which is encoded by a nucleic acid of about 321
nucleotides in length which begins at the amino terminal
Asp1 residue of the variable region through to Lys 107.
In the functional antibody, LM609 has a kappa light chain

10   constant region.

As with the Vitaxin nucleic acids, minor
modifications of these LM609 nucleotide sequences are
intended to be included as heavy and light chain LM609
encoding nucleic acids.   Such minor modifications are

15   included within the nucleic acids encoding LM609 heavy
and light chain polypeptides so long as the nucleic acids
or encoded polypeptides retain some or all of their
function as described.

Thus, the invention also provides a nucleic

20   acid encoding a LM609 heavy chain or functional fragment
wherein the nucleic acid encodes substantially the same
variable region amino acid sequence of monoclonal
antibody LM609 as that shown in Figure 2A (SEQ ID NO:6)
or a fragment thereof.   Similarly, the invention also

25   provides a nucleic acid encoding a LM609 light chain or
functional fragment wherein the nucleic acid encodes
substantially the same variable region amino acid
sequence of monoclonal antibody LM609 as that shown in
Figure 2B (SEQ ID NO:8) or a fragment thereof.

30   The invention further provides fragments of
LM609 heavy and light chain encoding nucleic acids
wherein such fragments consist substantially of the same

49

nucleotide or amino acid sequence as the variable region
of LM609 heavy or light chain polypeptides. The variable
region of the LM609 heavy chain polypeptide consists
essentially of nucleotides 1-351 and of amino acid

5   residues Glu1 to Ala117 of Figure 2A (SEQ ID NOS:5 and 6,
respectively). The variable region of the LM609 light
chain polypeptide consists essentially of nucleotides
1-321 and of amino acid residues Asp1 to Lys107 of Figure
2B (SEQ ID NOS:7 and 8, respectively). The termini of

10   such variable region encoding nucleic acids is not
critical so long as the intended purpose and function
remains the same. Such intended purposes and functions
include, for example, use for the production of
recombinant polypeptides or as hybridization probes for

15   heavy and light chain variable region sequences.

          Fragments additional to the variable region
nucleic acid fragments are provided as well. Such
fragments include, for example, nucleic acids consisting
substantially of the same nucleotide sequence as a CDR of

20   a LM609 heavy or light chain polypeptide. Sequences
corresponding to the LM609 CDRs include, for example,
those regions within the variable region which are
defined by Kabat et al., supra, and/or those regions
within the variable regions which are defined by Chothia

25   et al., supra, as well as those regions defined by
MacCallum et al., supra. The LM609 CDR fragments for
each of the above definitions correspond to the
nucleotides set forth below in Table 4. The nucleotide
sequence numbering is taken from the primary sequence

30   shown in Figures 2A and 2B (SEQ ID NOS:5 and 7) and
conforms to the definitions previously set forth in Table
1.

50

Table 4: LM609 CDR Nucleotide Residues

	Kabat	Chothia	MacCallum
V_H CDR1	91-105	76-96	88-105
V_H CDR2	148-198	157-168	139-177
V_H CDR3	295-318	298-315	288-315
V_L CDR1	70-102	76-96	88-108
V_L CDR2	148-168	148-156	136-165
V_L CDR3	265-291	271-288	265-288

Similarly, the LM609 CDR fragments for each of the above definitions correspond to the amino acid residues set forth below in Table 5. The amino acid residue numbering is taken from the primary sequence shown in Figures 2A and 2B (SEQ ID NOS:6 and 8) and conforms to the definitions set forth in Table 1.

Table 5: LM609 CDR Amino Acid Residues

	Kabat	Chothia	MacCallum
V_H CDR1	Ser31-Ser35	Gly26-Tyr32	Ser30-Ser35
V_H CDR2	Lys50-Gly66	Ser53-Gly56	Trp47-Tyr59
V_H CDR3	His99-Tyr106	Asn100-Ala105	Ala97-Ala105
V_L CDR1	Gln24-His34	Ser26-His32	Ser30-Tyr36
V_L CDR2	Tyr50-Ser56	Tyr50-Ser52	Leu46-Ile55
V_L CDR3	Gln89-Thr97	Ser91-His96	Gln89-His96

Nucleic acids encoding LM609 heavy and light chain polypeptides and fragments thereof are useful for a variety of diagnostic and therapeutic purposes. For example, the LM609 nucleic acids can be used to produce recombinant LM609 antibodies and functional fragments thereof having binding specificity and inhibitory activity against the integrin $\alpha_v\beta_3$. The antibody and functional fragments thereof can be used to determine the

51

presence or absence of $\alpha_v\beta_3$ in a sample to diagnose the
susceptibility or occurrence of an $\alpha_v\beta_3$-mediated disease.
Alternatively, the recombinant LM609 antibodies and
functional fragments thereof can be used for the
5   therapeutic treatment of $\alpha_v\beta_3$-mediated diseases or
pathological state. As with Vitaxin, recombinant LM609
and functional fragments thereof can be used to inhibit
the binding activity or other functional activities of
$\alpha_v\beta_3$ that are necessary for progression of the
10   $\alpha_v\beta_3$-mediated disease or pathological state.

The LM609 nucleic acids of the invention can
also be used to model functional equivalents of the
encoded heavy and light chain polypeptides. Such
functional equivalents can include, for example,
15   synthetic analogues or mimics of the encoded polypeptides
or functional fragments thereof. A specific example
would include peptide mimetics of the LM609 CDRs that
retain some or substantially the same binding or
inhibitory activity of LM609. Additionally, the LM609
20   encoding nucleic acids can be used to engineer and
produce nucleic acids which encode modified forms or
derivatives of the antibody LM609, its heavy and light
chain polypeptides and functional fragments thereof. As
described previously, such modified forms or derivatives
25   include, for example, non-mouse antibodies, their
corresponding heavy and light chain polypeptides and
functional fragments thereof which exhibit substantially
the same binding and inhibitory activity as LM609.

The invention also provides a method of
30   treating an $\alpha_v\beta_3$-mediated disease. The method consists of
administering an effective amount of Vitaxin, a LM609
grafted antibody, an enhanced antibody thereof, or a
functional fragment thereof under conditions which allow

52

binding to $\alpha_v\beta_3$. Also provided is a method of inhibiting
a function of $\alpha_v\beta_3$. The method consists of contacting $\alpha_v\beta_3$
with Vitaxin, a LM609 grafted antibody or a functional
fragment thereof under conditions which allow binding to
5   $\alpha_v\beta_3$.

As described previously, Vitaxin and LM609
grafted antibodies are monoclonal antibodies which
exhibit essentially all of the binding characteristics as
does its parental CDR-donor antibody LM609. These
10 characteristics include, for example, significant binding
specificity and affinity for the integrin $\alpha_v\beta_3$. The
Examples below demonstrate these binding properties and
further show that the binding of such antibodies to $\alpha_v\beta_3$
inhibits $\alpha_v\beta_3$ ligand binding and function. Thus, Vitaxin
15 and LM609 grafted antibodies are useful for a large
variety of diagnostic and therapeutic purposes directed
to the inhibition of $\alpha_v\beta_3$ function.

The integrin $\alpha_v\beta_3$ functions in numerous cell
adhesion and migration associated events. As such, the
20 dysfunction or dysregulation of this integrin, its
function, or of cells expressing this integrin, is
associated with a large number of diseases and
pathological conditions. The inhibition $\alpha_v\beta_3$ binding or
function can therefore be used to treat or reduce the
25 severity of such $\alpha_v\beta_3$-mediated pathological conditions.
Described below are examples of several pathological
conditions mediated by $\alpha_v\beta_3$, since the inhibition of at
least this integrin reduces the severity of the
condition. These examples are intended to be
30 representative and as such are not inclusive of all
$\alpha_v\beta_3$-mediated diseases. For example, there are numerous
pathological conditions additional to those discussed
below which exhibit the dysregulation of $\alpha_v\beta_3$ binding,

53

function or the dysregulation of cells expressing this
integrin and in which the pathological condition can be
reduced, or will be found to be reduced, by inhibiting
the binding $\alpha_v\beta_3$. Such pathological conditions which
5   exhibit this criteria, are intended to be included within
the definition of the term as used herein.

        Angiogenesis, or neovascularization, is the
process where new blood vessels form from pre-existing
vessels within a tissue. As described further below,
10 this process is mediated by endothelial cells expressing
$\alpha_v\beta_3$ and inhibition of at least this integrin, inhibits
new vessel growth. There are a variety of pathological
conditions that require new blood vessel formation or
tissue neovascularization and inhibition of this process
15 inhibits the pathological condition. As such,
pathological conditions that require neovascularization
for growth or maintenance are considered to be
$\alpha_v\beta_3$-mediated diseases. The extent of treatment, or
reduction in severity, of these diseases will therefore
20 depend on the extent of inhibition of neovascularization.
These $\alpha_v\beta_3$-mediated diseases include, for example,
inflammatory disorders such as immune and non-immune
inflammation, chronic articular rheumatism, psoriasis,
disorders associated with inappropriate or inopportune
25 invasion of vessels such as diabetic retinopathy,
neovascular glaucoma and capillary proliferation in
atherosclerotic plaques as well as cancer disorders.
Such cancer disorders can include, for example, solid
tumors, tumor metastasis, angiofibromas, retrolental,
30 fibroplasia, hemangiomas, Kaposi's sarcoma and other
cancers which require neovascularization to support tumor
growth. Additional diseases which are considered
angiogenic include psoriasis and rheumatoid arthritis as
well as retinal diseases such as macular degeneration.

54

Diseases other than those requiring new blood vessels
which are $\alpha_v\beta_3$-mediated diseases include, for example,
restenosis and osteoporosis.

Treatment of the $\alpha_v\beta_3$-mediated diseases can be
5   performed by administering an effective amount of
Vitaxin, a LM609 grafted antibody, an enhanced antibody
thereof, or a functional fragment thereof so as to bind
to $\alpha_v\beta_3$ and inhibit its function. Administration can be
performed using a variety of methods known in the art.
10   The choice of method will depend on the specific $\alpha_v\beta_3$-
mediated disease and can include, for example, the *in
vivo, in situ* and *ex vivo* administration of Vitaxin, a
LM609 grafted antibody or functional fragment thereof, to
cells, tissues, organs, and organisms. Moreover, such
15   antibodies or functional fragments can be administered to
an individual exhibiting or at risk of exhibiting an $\alpha_v\beta_3$-
mediated disease. Definite clinical diagnosis of an $\alpha_v\beta_3$-
mediated disease warrants the administration of Vitaxin,
a LM609 grafted antibody or a functional fragment
20   thereof. Prophylactic applications are warranted in
diseases where the $\alpha_v\beta_3$-mediated disease mechanisms
precede the onset of overt clinical disease. Thus,
individuals with familial history of disease and
predicted to be at risk by reliable prognostic indicators
25   can be treated prophylactically to interdict $\alpha_v\beta_3$-mediated
mechanisms prior to their onset.

Vitaxin, a LM609 grafted antibody, an enhanced
antibody thereof, or functional fragments thereof can be
administered in a variety of formulations and
30   pharmaceutically acceptable media for the effective
treatment or reduction in the severity of an $\alpha_v\beta_3$-mediated
disease. Such formulations and pharmaceutically
acceptable medias are well known to those skilled in the

55

art. Additionally, Vitaxin, a LM609 grafted antibody or
functional fragments thereof can be administered with
other compositions which can enhance or supplement the
treatment or reduction in severity of an $\alpha v\beta_3$-mediated
5   disease. For example, the coadministration of Vitaxin or
a LM609 grafted antibody to inhibit tumor-induced
neovascularization and a chemotherapeutic drug to
directly inhibit tumor growth is one specific case where
the administration of other compositions can enhance or
10   supplement the treatment of an $\alpha_v\beta_3$-mediated disease.

     Vitaxin, a LM609 grafted antibody or functional
fragments are administered by conventional methods, in
dosages which are sufficient to cause the inhibition of
$\alpha_v\beta_3$ integrin binding at the sight of the pathology.
15   Inhibition can be measured by a variety of methods known
in the art such as *in situ* immunohistochemistry for the
prevalence of $\alpha_v\beta_3$ containing cells at the site of the
pathology as well as include, for example, the observed
reduction in the severity of the symptoms of the
20   $\alpha_v\beta_3$-mediated disease.

     In vivo modes of administration can include
intraperitoneal, intravenous and subcutaneous
administration of Vitaxin, a LM609 grafted antibody or a
functional fragment thereof. Dosages for antibody
25   therapeutics are known or can be routinely determined by
those skilled in the art. For example, such dosages are
typically administered so as to achieve a plasma
concentration from about 0.01 µg/ml to about 100 µg/ml,
preferably about 1-5 µg/ml and more preferably about 5
30   µg/ml. In terms of amount per body weight, these dosages
typically correspond to about 0.1-300 mg/kg, preferably
about 0.2-200 mg/kg and more preferably about 0.5-20
mg/kg. Depending on the need, dosages can be

56

administered once or multiple times over the course of the treatment. Generally, the dosage will vary with the age, condition, sex and extent of the $\alpha_v\beta_3$-mediated pathology of the subject and should not be so high as to

5   cause adverse side effects. Moreover, dosages can also be modulated by the physician during the course of the treatment to either enhance the treatment or reduce the potential development of side effects. Such procedures are known and routinely performed by those skilled in the

10 art.

The specificity and inhibitory activity of Vitaxin, LM609 grafted antibodies, an enhanced antibody thereof and functional fragments thereof allow for the therapeutic treatment of numerous $\alpha_v\beta_3$-mediated diseases.

15 Such diseases include, for example, pathological conditions requiring neovascularization such as tumor growth, and psoriasis as well as those directly mediated by $\alpha_v\beta_3$ such as restenosis and osteoporosis. Thus, the invention provides methods as well as Vitaxin and LM609

20 grafted antibody containing compositions for the treatment of such diseases.

Throughout this application various publications are referenced within parentheses. The disclosures of these publications in their entireties are

25 hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

It is understood that modifications which do not substantially affect the activity of the various

30 embodiments of this invention are also included within the definition of the invention provided herein.

57

Accordingly, the following examples are intended to
illustrate but not limit the present invention.

EXAMPLE I

Isolation and Characterization of LM609
Encoding Nucleic Acids

5

This Example shows the cloning and sequence
determination of LM609 encoding nucleic acids.

LM609 is directed against the human vitronectin
receptor, integrin $\alpha_v\beta_3$. $\alpha_v\beta_3$ is highly upregulated in
10   melanoma, glioblastoma, and mammary carcinoma and plays a
role in the proliferation of M21 melanoma cells both in
vitro and in vivo. $\alpha_v\beta_3$ also plays a role in
angiogenesis, restenosis and the formation of granulation
tissue in cutaneous wounds. LM609 has been shown to
15   inhibit the adhesion of M21 cells to vitronectin as well
as prevent proliferation of M21 cells in vitro. Thus,
grafting of LM609 could result in a clinically valuable
therapeutic agent.

cDNA Synthesis of LM609 Variable Regions: For
20   cDNA synthesis, total RNA was prepared from 10^8 LM609
hybridoma cells using a modification of the method
described by Chomczynski and Sacchi (Chomczynski and
Sacchi, *Analyt. Biochem.* 162:156 (1987)). LM609 variable
(V) region genes were cloned by reverse
25   transcription-polymerase chain reaction (RT-PCR) and cDNA
was synthesized using BRL Superscript kit. Briefly, 5 µg
of total cellular RNA, 650 ng oligo dT and H_2O were
brought to a total volume of 55 µl. The sample was
heated to 70°C for 10 min and chilled on ice. Reaction
30   buffer was added and the mixture brought to 10 mM DTT and

58

1 mM dNTPs and heated at 37°C for 2 minutes. 5 μl (1000

units) reverse transcriptase was added and incubated at

37°C for 1 hour and then chilled on ice.

        All oligonucleotides were synthesized by

5   β-cyanoethyl phosphoramidite chemistry on an ABI 394 DNA

    synthesizer. Oligonucleotides used for PCR amplification

    and routine site-directed mutagenesis were purified using

    oligonucleotide purification cartridges (Applied

    Biosystems, Foster City, CA). Forward PCR primers were

10 designed from N-terminal protein sequence data generated

    from purified LM609 antibody. The forward PCR primers

    contained sequences coding for the first six amino acids

    in each antibody variable chain (protein sequenced at San

    Diego State University). The sequence of the light chain

15 forward PCR primer (997) was 5'-GCC CAA CCA GCC ATG GCC

    GAT ATT GTG CTA ACT CAG-3' (SEQ ID NO:19) whereas the

    light chain reverse PCR primer (734) was 5'-AC AGT TGG

    TGC AGC ATC AGC-3' (SEQ ID NO:20) used. This reverse

    primer corresponds to mouse light chain kappa amino acid

20 residues 109-115. The sequence of the heavy chain

    forward PCR primer (998) was 5'-ACC CCT GTG GCA AAA GCC

    GAA GTG CAG CTG GTG GAG-3' (SEQ ID NO:21). Heavy chain

    reverse PCR primer 733: 5'-GA TGG GGG TGT CGT TTT GGC-3'

    SEQ ID NO:22). The PCR primers also contain regions of

25 homology with specific sequences within the

    immunoexpression vector.

        V_L and V_H chains were amplified in two separate

    50 μl reaction mixtures containing 2 μl of the cDNA-RNA

    heteroduplex, 66.6 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 0.2

30 mM of each four dNTPs, 10 mM 2-mercaptoethanol, 0.25

    units Taq polymerase (Boehringer-Mannheim, Indianapolis,

    IN) and 50 pmoles each of primers 997 and 734 and 998 and

59

733, respectively. The mixtures were overlaid with
mineral oil and cycled for two rounds of PCR with each
cycle consisting of 30 seconds at 94°C (denature), 30
seconds at 50°C (anneal), and 30 seconds at 72°C
5   (synthesis). This reaction was immediately followed by
30 cycles of PCR consisting of 30 seconds at 94°C
(denature), 30 seconds at 55°C (anneal), and 30 seconds at
72°C (synthesis) followed by a final synthesis reaction
for 5 minutes at 72°C. The reaction products were pooled,
10   extracted with CHCl$_3$ and ethanol precipitated.

Amplified products were resuspended in 20 µl TE
buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and
electrophoresed on a 5% polyacrylamide gel. Bands
migrating at expected molecular weights of V_H and V_L were
15   excised, chemically eluted from the gel slice, extracted
with organic solvents and ethanol precipitated.

Cloning of amplified V_H and V_L genes into M13
phage immunoexpression vector: The amplified V region
gene products were sequentially cloned into the phage
20   immunoexpression vector by hybridization mutagenesis
(Near, R. *Biotechniques* 12:88 (1992); Yelton et al., *J.
Immunol.* 155:1994-2003 (1995)). Introduction of the
amplified V_L and V_H sequences by hybridization mutagenesis
positions the antibody sequences in frame with the
25   regulatory elements contained in the M13 vector required
for efficient Fab expression. One advantage of this
technique is that no restriction endonuclease sites need
to be incorporated into the V_L or V_H gene sequences for
cloning as is done with conventional DNA ligation
30   methods.

To perform the cloning, 400 ng each of the
double-stranded amplified products were first

phosphorylated with polynucleotide kinase. 100 ng of the
phosphorylated LM609 V_L product was mixed with 250 ng of
uridinylated BS11 phage immunoexpression vector,
denatured by heating to 90°C and annealed by gradual

5   cooling to room temperature. BS11 is an M13
immunoexpression vector derived from M13 IX and encodes
CH_1 of murine IgG1 and murine kappa light chain constant
domain (Huse, W.D. In: Antibody Engineering: A Practical
Guide, C.A.K. Borrebaeck, ed. W.H. Freeman and Co.,

10 Publishers, New York, pp. 103-120 (1991)). Nucleotide
sequences included in the PCR amplification primers
anneal to complementary sequences present in the single-
stranded BS11 vector. The annealed mixture was fully
converted to a double-stranded molecule with T4 DNA

15 polymerase plus dNTPs and ligated with T4 ligase. 1 µl
of the mutagenesis reaction was electroporated into E.
coli strain DH10B, titered onto a lawn of XL-1 E. coli
and incubated until plaques formed. Plaque lift assays
were performed as described using goat anti-murine kappa

20 chain antibody conjugated to alkaline phosphatase (Yelton
et al, supra; Huse, W.D., supra). Fifteen murine light
chain positive M13 phage clones were isolated, pooled and
used to prepare uridinylated vector to serve as template
for hybridization mutagenesis with the PCR amplified

25 LM609 V_H product.

Clones expressing functional murine LM609 Fab
were identified by binding to purified $\alpha_v\beta_3$ by ELISA.
Briefly, Immulon II ELISA plates were coated overnight
with 1 µg/ml (100 ng/well) $\alpha_v\beta_3$ and nonspecific sites

30 blocked for two hours at 27°C. Soluble Fabs were prepared
by isolating periplasmic fractions of cultures of E. coli
strain MK30-3 (Boehringer Mannheim Co.) infected with the
Fab expressing M13 phage clones. Periplasm fractions

61

were mixed with binding buffer 100 mM NaCl, 50 mM Tris pH
7.4, 2mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.02% NaN_3, 1 mg/ml
BSA and incubated with immobilized $\alpha_v\beta_3$ for two hours at
27°C. Plates were washed with binding buffer and bound
5   Fab detected with goat anti-murine kappa chain antibody
conjugated to alkaline phosphatase. Four $\alpha_v\beta_3$ reactive
clones were identified: muLM609M13 12, 29, 31 and 69.
MuLM609M13 12 and 29 gave the strongest signals in the
ELISA assay. DNA sequence analysis showed that clones
10 muLM609M13 12, 31 and 69 all had identical light chain
sequence and confirmed the previously determined
N-terminal amino acid sequence of purified LM609 light
chain polypeptide. All four clones had identical V_H DNA
sequence and also confirmed the previously determined
15 N-terminal amino acid sequence of purified LM609 heavy
chain polypeptide.

        To further characterize the binding activity of
each clone, soluble Fab fractions were prepared from 50
ml cultures of *E. coli* strain MK30-3 infected with clones
20 12 and 29 and evaluated for binding to $\alpha_v\beta_3$ in a
competitive ELISA with LM609 IgG. The results of this
ELISA are shown in Figure 3. Clone muLM609M13 12 was
found to inhibit LM609 IgG binding (at LM609 IgG
concentrations of 1 ng/ml and 5 ng/ml) to $\alpha_v\beta_3$ in a
25 concentration dependent manner at periplasm titers
ranging from neat to 1:80. Clone muLM609M13 12 was
plaque purified and both the V region heavy and light
chain DNA sequences again determined. Complete DNA
sequence of the final clone, muLM609M13 12-5, is shown in
30 Figures 2A and 2B.

62

EXAMPLE II

Construction of Vitaxin: A CDR Grafted
LM609 Functional Fragment

One goal of grafting antibodies is to preserve

5   antibody specificity and affinity when substituting

non-human CDRs into a human antibody framework. Another

goal is to minimize the introduction of foreign amino

acid sequences so as to reduce the possible antigenicity

with a human host. This Example describes procedures for

10   accomplishing both of these goals by producing libraries

of grafted antibodies which represent all

possible members which exhibit the highest affinities for

the desired antigen.

The above library was constructed in *E. coli*

15   wherein the possible CDR and framework changes were

incorporated using codon-based mutagenesis (Kristensson

et al., In: Vaccines 95. Cold Spring Harbor Laboratory

Press. Cold Spring Harbor, NY (1995); Rosok et al., *J.*

Biol. Chem. (271:22611-22613 (1996)). Using these

20   procedures, a library was constructed and a functionally

active humanized anti-$\alpha_v\beta_3$-inhibitory antibody was

identified.

For the construction of one grafted form of

LM609, human framework sequences showing the highest

25   degree of identity to the murine LM609 V region gene

sequences were selected for receiving the LM609 CDRs.

Human heavy chain V region M72 'CL (HHC30Q, HC Subgroup

3, Kabat et al., *supra*) had 88% identity to frameworks 1,

2 and 3 of LM609 heavy chain and human light chain V

30   region LS1 'CL (HKL312, Kappa subgroup 3, Kabat et al.,

supra) had 79% identity to frameworks 1, 2 and 3 of LM609

63

light chain.   Murine LM609 CDR sequences, as defined by
Kabat et al., *supra* were grafted onto the human
frameworks.   Residues predicted to be buried that might
affect the structure and therefore the binding properties

5   of the original murine combining site were taken into
consideration when designing possible changes (Singer et
al., *supra*; Padlan, E.A. *Mol. Immunol.* 28:489-498
(1991)).   This analysis of framework residues considered
to be important for preserving the specificity and

10   affinity of the combining site revealed only a few
differences.   For example, in the heavy chain sequence,
the predicted buried residues displayed 100% identity.
Of particular note is that Arg16 in human heavy chain V
region M72 'CL is a relatively uncommon residue among

15   human chains.   However, this residue was also found to be
present in LM609 V_H and therefore was retained.
Similarly, Arg19 in LM609 is a relatively rare residue
among murine heavy chains but it is found to occur in M72
'CL and was therefore retained.   In the light chain

20   sequences, two nonidentical buried residues were
identified between LM609 and LS1 'CL framework regions at
positions 49 and 87.   These two positions were therefore
incorporated into the grafted antibody library as both
human and murine alternatives.

25   Full-length grafted V region genes were
synthesized by PCR using long overlapping
oligonucleotides.   Light chain oligonucleotides
containing mixed amino acid residues at positions 49 and
87 were synthesized as described in Glaser et al. (*J.*

30   *Immunol.* 149:3903-3913 (1992)) and as illustrated in the
oligonucleotides represented as V_L oligo3 and V_L oligo4.
(SEQ ID NOS:16 and 17, respectively).   All long
oligonucleotides were gel purified.

64

Grafted LM609 heavy and light chain V regions
were constructed by mixing 5 overlapping oligonucleotides
at equimolar concentrations, in the presence of annealing
PCR primers. The heavy chain oligonucleotides map to the
5   following nucleotide positions: V_H oligonucleotide 1 (V_H
oligo1), nucleotides (nt) 1-84; (SEQ ID NO:9); V_H oligo2,
nt 70-153, (SEQ ID NO:10); V_H oligo3, nt 138-225 (SEQ ID
NO:11); V_H oligo4, nt 211-291 (SEQ ID NO:12); V_H oligo5,
nt 277-351 (SEQ ID NO:13). Similarly, the Vitaxin light
10 chain oligonucleotides map to the following nucleotide
positions: V_L oligonucleotide 1 (V_L oligo1), nucleotides
(nt) 1-87; (SEQ ID NO:14); V_L oligo2, nt 73-144, (SEQ ID
NO:15); V_L oligo3, nt 130-213 (SEQ ID NO:16); V_L oligo4,
nt 199-279 (SEQ ID NO:17); V_L oligo5, nt 265-321 (SEQ ID
15 NO:18). The nucleotide sequences of oligonucleotides
used to construct grafted LM609 heavy and light chain
variable regions are shown in Table 6. Codon positions
49 and 87 in V_L oligo3, and V_L oligo4 represent the
randomized codons. The annealing primers contained at
20 least 18 nucleotide residues complementary to vector
sequences for efficient annealing of the amplified V
region product to the single-stranded vector. The
annealed mixture was fully converted to a double-stranded
molecule with T4 DNA polymerase plus dNTPs and ligated
25 with T4 ligase.

65

Table 6: Oligonucleotides Used to Construct Grafted LM609 Heavy and Light Chain Variable Regions

CAGGTGCAGC TGGTGGAGTC TGGGGGAGGC GTTGTGCAGC CTGGAAGGTC CCTGAGACTC

TCCTGTGCAG CCTCTGGATT CACC   SEQ ID NO: 9

5   AACTTTTGCG ACCCACTCCA GACCCTTGCC CGGAGCCTGG CGAACCCAAG ACATGTCATA

GCTACTGAAG GTGAATCCAG AGGC   SEQ ID NO: 10

TGGGTCGCAA AAGTTAGTAG TGGTGGTGGT AGCACCTACT ATTTAGACAC TGTGCAGGGC

CGATTCACCA TCTCCAGAGA CAATAGT   SEQ ID NO: 11

TGCACAGTAA TACACGGCTG TGTCCTCGGC TCTCAGAGAG TTCATTTGCA GGTATAGGGT

10   GTTCTTACTA TTGTCTCTGG A   SEQ ID NO: 12

GTGTATTACT GTGCAAGACA TAACTACGGC AGTTTTGCTT ACTGGGGCCA AGGGACTACA

GTGACTGTTT CTAGT   SEQ ID NO: 13

GAGATTGTGC TAACTCAGTC TCCAGCCACC CTGTCTCTCA GCCCAGGAGA AAGGGCGACT

CTTTCCTGCC AGGCCAGCCA AAGTATT   SEQ ID NO: 14

15   GATGAGAAGC CTTGGGGCTT GACCAGGCCT TTGTTGATAC CAGTGTAGGT GGTTGCTAAT

ACTTTGGCTG GC   SEQ ID NO: 15

CCAAGGCTTC TCATCWASTA TCGTTCCCAG TCCATCTCTG GGATCCCCGC CAGGTTCAGT

GGCAGTGGAT CAGGGACAGA TTTC   SEQ ID NO: 16

GCTGCCACTC TGTTGACAGW AATAGACTGC AAAATCTTCA GGCTCCAGAC TGGAGATAGT

20   GAGGGTGAAA TCTGTCCCTG A   SEQ ID NO: 17

CAACAGAGTG GCAGCTGGCC TCACACGTTC GGAGGGGGGA CCAAGGTGGA AATTAAG

                                              SEQ ID NO: 18

        To generate the library, a portion of the
mutagenesis reaction (1 μl) was electroporated into $E.$
25   $coli$ strain DH10B (BRL), titered onto a lawn of XL-1
(Stratagene, Inc.) and incubated until plaques formed.
Replica filter lifts were prepared and plaques containing
V_H gene sequences were screened either by hybridization
with a digoxigenin-labeled oligonucleotide complementary
30   to LM609 heavy chain CDR 2 sequences or reactivity with
7F11-alkaline phosphatase conjugate, a monoclonal
antibody raised against the decapeptide sequence Tyr Pro
Tyr Asp Val Pro Asp Tyr Ala Ser (SEQ ID NO:28) appended
to the carboxy terminus of the vector CH_1 domain (Biosite,

66

Inc., San Diego, CA). Fifty clones that were double-
positive were pooled and used to prepare uridinylated
template for hybridization mutagenesis with the amplified
grafted LM609 V_L product.

5   The mutagenesis reaction was performed as
described above with the V_H oligonucleotides except that
the V_L oligonucleotides 1 to 5 were employed (SEQ ID
NOS:14 to 18, respectively). The reaction was
electroporated into *E. coli* strain DH10B and filter lifts
10 probed with either goat anti-human kappa chain antibody
conjugated to alkaline phosphatase or a goat anti-human
Fab antibody using an alkaline phosphatase conjugated
rabbit anti-goat secondary reagent for detection.
Positive clones co-expressing both V_H and V_L gene
15 sequences were selected (160 total) and used to infect *E.
coli* strain MK30-3 for preparing soluble Fab fragments.

         The soluble Fab fragments were screened for
binding to $\alpha_v\beta_3$ in an ELISA assay. Four clones that were
shown from the ELISA to strongly bind $\alpha_v\beta_3$ were identified
20 and further characterized. These clones were termed
huLM609M13-34, 54, 55 and 145. All four clones were
plaque purified and three independent subclones from each
clone was used to prepare Fab fragments for additional
binding analysis to $\alpha_v\beta_3$ by ELISA.

25   In this additional ELISA, duplicate plates were
coated with $\alpha_v\beta_3$ ligand and incubated with the huLM609
periplasmic samples. In one plate, bound huLM609 Fab was
detected with goat anti-human kappa chain antibody
conjugated to alkaline phosphatase and in the other plate
30 bound huLM609 Fab was detected with 7F11-alkaline
phosphatase conjugate, the monoclonal antibody

67

recognizing the decapeptide tag. Subclones huLM609M13-
34-1, 2 and 3 and huLM609M13-145-1, 2 and 3 all yielded
double positive signals indicating that the Fabs contain
functional V_H and V_L polypeptides. These results were
5 confirmed in an ELISA assay on M21 cells, a cell line
that expresses the integrin $\alpha_v\beta_3$.

DNA sequence analysis of subclones huLM609M13-
34-3 and huLM609M13-145-3 revealed mutations introduced
into the library by errors due to oligonucleotide
10 synthesis or by errors arising during PCR amplification.
These mutations were corrected in clone huLM609M13-34-3
by site-directed mutagenesis. In the light chain
sequence the following corrections were made: His36 to
Tyr36 and Lys18 to Arg18. In the heavy chain sequence
15 the following corrections were made: Glu1 to Gln1, Asn3
to Gln3, Leu11 to Val11. Additionally, during the
construction of LM609 grafted molecules, residue 28 from
the heavy chain was considered to be a non-critical
framework residue and the human residue (Thr28) was
20 retained. Subsequently, however, it has been determined
that residue 28 can be considered part of the CDR.
Therefore, residue 28 was converted to the corresponding
mouse residue at that position (Ala28) using site
directed mutagenesis with the oligonucleotide 5'-GCT ACT
25 GAA GGC GAA TCC AGA G-3' (SEQ ID NO:29). This change was
later determined to not provide benefit over the human
framework threonine at this site, and the threonine was
retained. The final grafted LM609 clone was designated
huLM609M13 1135-4 and is termed herein Vitaxin. The DNA
30 sequence of clone Vitaxin is shown in Figures 2A and 2B.

68

EXAMPLE III

Functional Characterization of Vitaxin

This Example shows the characterization of
Vitaxin's binding specificity, affinity and functional
5   activity in a number of *in vitro* binding and cell
adhesion assays.

The binding specificity of Vitaxin for the
integrin $\alpha_v\beta_3$ was initially assessed by measuring binding
to $\alpha_v\beta_3$ and its crossreactivity to other α_v- or β_3-
10 containing integrins. Specifically, binding specificity
was assessed by measuring binding to $\alpha_{IIb}\beta_3$, the major
integrin expressed on platelets, and to $\alpha_v\beta_5$, an integrin
found prevalent on endothelial cells and connective
tissue cell types.

15   Briefly, to determine crossreactivity,
integrins were coated onto an ELISA plate and a series of
antibody dilutions were measured for Vitaxin binding
activity against $\alpha_v\beta_3$ and the other integrins. The
integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were isolated by affinity
20 chromatography as described by Cheresh (1987), *supra*, and
Cheresh and Spiro (1987), *supra*. $\alpha_{IIb}\beta_3$ was purchased from
CalBiochem. Briefly, an LM609 affinity column (Cheresh
and Spiro (1987), *supra*) was used to isolate $\alpha_v\beta_3$ from an
octylglucoside human placental lysate, whereas an anti-α_v
25 affinity column was used to isolate $\alpha_v\beta_5$ from the
$\alpha_v\beta_3$-depleted column flow through. Antibody binding
activity was assessed by ELISA using a goat anti-human
IgG-alkaline phosphatase conjugate. As a control, a
purified human IgG$_1$ antibody was used since Vitaxin
30 contains a human IgG$_1$ backbone.

69

The results of this assay are shown in Figure
4A and reveal that Vitaxin specifically binds to $\alpha_v\beta_3$ with
high affinity. There was no detectable binding to the
other α_v- or β_3-containing integrins at antibody

5   concentrations over 1.0 mg/ml.

In a further series of binding studies, the
binding affinity and specificity was assessed in a
competitive binding assay with the parental LM609
antibody against $\alpha_v\beta_3$. Competitive binding was measured

10   in an ELISA assay as described above with LM609 being the
labeled antibody. Binding of LM609 was determined in the
presence of increasing concentrations of Vitaxin
competitor. Alternatively, the control competitor
antibody was again a human IgG_1.

15   The results of this competition are presented
in Figure 4B and show that specific inhibition of LM609
binding can be observed at Vitaxin concentrations of over
0.1 µg/ml. Almost complete inhibition is observed at
Vitaxin concentrations greater than 100 µg/ml. This

20   level of competitive inhibition indicates that the
parental monoclonal antibody LM609 and the grafted
version Vitaxin exhibit essentially identical
specificity.

Binding affinity and specificity were also

25   assessed by measuring the inhibitory activity of Vitaxin
on $\alpha_v\beta_3$ binding to fibrinogen. For these studies, $\alpha_v\beta_3$ was
plated onto ELISA plates as described above for the
Vitaxin/$\alpha_v\beta_3$ binding studies. Inhibitory activity of
Vitaxin was determined by measuring the amount of bound

30   biotinylated fibrinogen in the presence of increasing
concentrations of Vitaxin or control antibody. Briefly,
fibrinogen was purchased from CalBiochem and biotinylated

70

with N-hydroxysuccinimidobiotin as described by the
manufacturer (Pierce Life Science and Analytical
Research). Streptavidin alkaline phosphatase was used to
detect the bound fibrinogen.

5   The results of this assay are presented in
Figure 4C and reveal a specific binding inhibition at
Vitaxin concentrations higher than about 0.1 μg/ml.
These results, combined with those presented above
showing specific binding of Vitaxin to $\alpha_v\beta_3$ and
10   competitive inhibition of LM609, demonstrate that Vitaxin
maintains essentially all of the binding characteristics
and specificity exhibited by the parental murine
monoclonal antibody LM609. Described below are
additional functional studies which corroborate these
15   conclusions based on *in vitro* binding assays.

          Additional functional studies were performed to
further assess the specificity of Vitaxin binding. These
studies were directed to the inhibition of integrin $\alpha_v\beta_3$
binding in cell adhesion assays. Endothelial cell
20   adhesion events are an important component in the
angiogenic process and inhibition of $\alpha_v\beta_3$ is known to
reduce the neovascularization of tumors and thereby
reduce the rate of tumor growth. The inhibition of
$\alpha_v\beta_3$-mediated cell attachment by Vitaxin in these assays
25   is indicative of the inhibitory activity expected when
this antibody is used *in situ* or *in vivo*.

          Briefly, $\alpha_v\beta_3$-positive M21 melanoma cells grown
in RPMI containing 10% FBS were used for these cell
binding assays. Cells were released from the culture
30   dish by trypsinization and re-suspended in adhesion
buffer at a concentration of 4×10^5 cells/ml (see below).
Vitaxin, LM609 or purified human IgG_1 (control antibody),

71

were diluted to the desired concentration in 250 µl
adhesion buffer (10 mM Hepes, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 0.2
mM MnCl$_2$, and 1% BSA in Hepes buffered saline at pH 7.4)
and added to wells of a 48-well plate precoated with

5   fibrinogen. The fibrinogen was isolated as described
above. Each well was coated with 200 μl fibrinogen at a
concentration of 10 μg/ml for 1 hour at 37°C. For the
assay, an equal volume of cells (250 µl) containing
Vitaxin, LM609 or isotype matched control antibody was

10   added to each of the wells, mixed by gentle shaking and
incubated for 20 minutes at 37°C. Unbound cells were
removed by washing with adhesion buffer until no cells
remained in control wells coated with BSA alone. Bound
cells were visualized by staining with crystal violet

15   which was subsequently extracted with 100 μl acetic acid
(10%) and quantitated by determining the absorbance of
the solubilized dye at 560 nm.

The results of this assay are shown in Figure
5A and reveal that both Vitaxin and parental antibody

20   LM609 inhibit M21 cell adhesion to fibrinogen over the
same concentration range. The inhibitory concentration
for 50% maximal adhesion was calculated to be about 50
ng/ml. Specificity of Vitaxin was shown by the lack of
inhibition observed by the control IgG$_1$ antibody.

25   In addition to the above cell adhesion results,
the inhibitory activity of Vitaxin was also tested in an
endothelial cell migration assay. In this regard, the
transwell cell migration assay was used to assess the
ability of Vitaxin to inhibit endothelial cell migration

30   (Choi et al., J. Vascular Surg., 19:125-134 (1994) and
Leavesly et al., J. Cell Biol, 121:163-170 (1993)).

72

Briefly, human umbilical vein endothelial cells in log phase and at low passage number were harvested by gentle trypsinization, washed and resuspended at a concentration of 2 x 10^6 cells/ml in 37°C HBS containing

5   1% BSA (20 mM HEPES, 150 mM NaCl, 1.8 mM CaCl$_2$, 1.8 mM MgCl$_2$, 5 mM KCl, and 5 mM glucose, pH 7.4). Antibodies (Vitaxin, LM609, and IgG$_1$ control) were diluted to 10 µg/ml from stock solutions. Antibodies were added to cells in a 1:1 dilution (final concentration of

10   antibodies = 5 µg/ml; final concentration of cells = 1 x 10^6 cells/ml) and incubated on ice for 10 - 30 minutes. The cell/antibody suspensions (200 µl to each compartment) were then added to the upper compartments of a Transwell cell culture chamber (Corning Costar), the

15   lower compartments of which had been coated with 0.5 ml of 10 µg/ml vitronectin (in HBS). Vitronectin serves as the chemoattractant for the endothelial cells. The chambers were placed at 37°C for 4 hours to allow cell migration to occur.

20   Visualization of cell migration was performed by first removing the remaining cells in the upper compartment with a cotton swab. Cells that had migrated to the lower side of the insert were stained with crystal violet for 30 minutes, followed by solubilization in

25   acetic acid and the absorbance of the dye was measured at a wavelength of 550 nm. The amount of absorbance is directly proportional to the number of cells that have migrated from the upper to the lower chamber. The results of the assay are presented in Figure 7B. Both

30   Vitaxin and the parental antibody LM609 yielded essentially identical inhibitory results. Specifically, Vitaxin and LM609 inhibited about 60% of the vitronectin-induced migration of endothelial cells compared to the IgG$_1$ control and to a sample with no inhibitor.

73

EXAMPLE IV

Vitaxin-Mediated Inhibition of $\alpha_v\beta_3$ In Animal Models

This Example describes the inhibition of tumor
growth by Vitaxin in two animal models. Tumor growth was
5   inhibited by inhibiting at least $\alpha_v\beta_3$-mediated
neovascularization with Vitaxin.

The first model measures angiogenesis in the
chick chorioallantoic membrane (CAM). This assay is a
well recognized model for *in vivo* angiogenesis because
10   the neovascularization of whole tissue is occurring.
Specifically, the assay measures growth factor induced
angiogenesis of chicken CAM vessels growing toward the
growth factor-impregnated filter disk or into the tissue
grown on the CAM. Inhibition of neovascularization is
15   based on the amount and extent of new vessel growth or on
the growth inhibition of tissue on the CAM. The assay
has been described in detail by others and has been used
to measure neovascularization as well as the
neovascularization of tumor tissue (Ausprunk et al., <u>Am.</u>
20   <u>J. Pathol.</u>, 79:597-618 (1975); Ossonski et al. <u>Cancer</u>
<u>Res.</u>, 40:2300-2309 (1980); Brooks et al. <u>Science</u>,
264:569-571 (1994a) and Brooks et al. <u>Cell</u>, 79:1157-1164
(1994b).

Briefly, for growth factor induced angiogenesis
25   filter disks are punched from #1 Whatman Qualitative
Circles using a skin biopsy punch. Disks are first
sterilized by exposure to UV light and then saturated
with varying concentrations of TNF-α or HBSS as a
negative control (for at least 1 hour) under sterile
30   conditions. Angiogenesis is induced by placing the
saturated filter disks on the CAMs.

74

Inhibition of angiogenesis is performed by
treating the embryos with various amounts of Vitaxin and
controls (antibody or purified human IgG$_1$). The
treatments are performed by intravenous injection
5   approximately 24 hours after disk placement. After 48
hours, CAMs are dissected and angiogenesis is scored on a
scale of 1-4. HBSS saturated filter disks are used as
the negative control, representing angiogenesis that may
occur in response to tissue injury in preparing CAMs,
10   and, values for these CAMS are subtracted out as
background. Purified human IgG$_1$ is used as the negative
control for injections since Vitaxin is of the human IgG$_1$
subclass. Vitaxin was found to inhibit TNF-α induced
angiogenesis in a dose dependent manner. Maximal
15   inhibition occurred with a single dose of Vitaxin at 300
μg which resulted in greater than 80% inhibition compared
to the human IgG$_1$ control.

In addition to the above described CAM assay
using growth factor-induced neovascularization,
20   additional studies were performed utilizing tumor-induced
neovascularization. For these assays, angiogenesis was
induced by transplantating of $\alpha_v\beta_3$-negative tumor
fragments into the CAMs. The use of $\alpha_v\beta_3$-negative tumor
fragments ensures that any inhibition of tumor growth is
25   due to the inhibition of $\alpha_v\beta_3$-mediated neovascularization
by CAM-derived endothelial cells and not to adhesion
events mediated by $\alpha_v\beta_3$ present on the tumor cells.

Inhibition of tumor growth was assessed by
placing a single cell suspension of FG (8 x 10^6 cells,
30   pancreatic carcinoma) and HEp-3 cells (5 x 10^5 cells,
laryngeal carcinoma) onto CAMs in 30 μl. One week later,
tumors are removed and cut into approximately 50 mg
fragments at which time they are placed onto new CAMs.

75

After 24 hours of this second placement embryos are injected intravenously with Vitaxin or human IgG_1 as a negative control. The tumors are allowed to grow for about 7 days following which they are removed and

5   weighed.

The results of Vitaxin treatment on the neovascularization of tumors is shown in Figure 6A. The data is expressed as a mean change in tumor weight and demonstrate that Vitaxin is able to inhibit the growth of

10   $\alpha_v\beta_3$-negative tumors such as FG and HEp-3 tumor fragments. More specifically, there was a mean weight change for Vitaxin treated FG tumor fragments of -5.38 whereas a change of -11.0 was observed for Vitaxin treated HEp-3 tumors. The IgG_1 controls exhibited positive mean weight

15   changes of 25.29 and 28.5 for the FG and HEp-3 tumor fragments, respectively. These results were obtained following a single intravenous injection.

In a second animal model, the inhibition of Vx2 carcinoma cells in rabbits was used as a measure of

20   Vitaxin's inhibitory effect on tumors. The Vx2 carcinoma is a transplantable carcinoma derived from a Shope virus-induced papilloma. It was first described in 1940 and has since been used extensively in studies on tumor invasion, tumor-host interactions and angiogenesis. The

25   Vx2 carcinoma is fibrotic in nature, highly aggressive, and exhibits features of an anaplastic type carcinoma. Propagation of Vx2 tumor is accomplished through serial transplantation in donor rabbits. Following subcutaneous transplantation, it has been reported that after an

30   initial inflammatory reaction, host repair mechanisms set in between days 2 and 4. This repair mechanism is characterized by the formation of new connective tissue and the production of new capillaries. The newly formed

76

capillaries are restricted to the repair zone at day 4,
however, by day 8 they have extended to the outer region
of the tumor. These characteristics and the
pharmacokinetics of Vitaxin in rabbits were used to
5   determine initial doses and scheduling of treatments for
these experiments. The elimination half life of Vitaxin
in animal serum dosed at 1, 5, and 10 mg/kg was found to
be 38.9, 60.3, and 52.1 hours, respectively.

        Growth of Vx2 tumors in the above animal model
10 was used to study the effect of Vitaxin after early
administration on primary tumor growth in rabbits
implanted subcutaneously with Vx2 carcinoma. Briefly,
Vx2 tumors (50 mg) were transplanted into the inner thigh
of rabbits through an incision between the skin and
15 muscle. Measurements of the primary tumor were taken
throughout the experiment through day 25. At day 28
after the transplantation animals were sacrificed and
tumors were excised and weighed. By day 28, tumors
became extremely irregular in shape and as a result,
20 measurements became difficult and were not reflective of
tumor volume. Therefore measurements were assessed only
through day 25.

        In a first study, rabbits were treated starting
at day 1 post tumor implantation with 5 and 1 mg/kg
25 Vitaxin every four days for 28 days for a total of 7
doses). In both groups, inhibition of tumor growth was
observed. In a second series of studies, rabbits were
treated beginning at day 7 post tumor implantation as
described above for a total of 5 doses. Inhibition of
30 tumor growth was also observed.

        It should be noted that administering a grafted
antibody as a repeat dose treatment to rabbits might

77

generate an immune response that can have a neutralizing
effect on Vitaxin thus potentially comprising efficacy.
Preliminary data suggest that approximately 25-50% of the
animals develop such a response.

5   The results of each of the Vitaxin treatments
described above is shown in Figure 6B and 6C. In the
rabbits receiving treatments on day 1, inhibition of
tumor growth was observed in both the 1 mg/kg and the 5
mg/kg dosing groups compared to the control PBS treated
10 control. Specifically, a growth inhibition of about 67
and 80% was observed, respectively, as measured by the
mean tumor weight. A lesser degree of inhibition was
observed in animals that began Vitaxin treatment on day 7
post implantation. These results are shown in Figure 6C.
15 In all cases, inhibition of tumor growth was not see at
Vitaxin concentrations lower than 0.2 mg/kg.

                          EXAMPLE V
       Construction of LM609 Grafted Functional Antibody
                          Fragments

20   This Example shows the construction of
functional LM609 grafted antibody fragments in which only
the CDRs have been transferred from the LM609 donor
antibody to a human acceptor framework.

            CDR grafting of LM609 to produce a functional
25 antibody fragment was accomplished by the methods set
forth below. These procedures are applicable for the CDR
grafting of essentially any donor antibody where amino
acid residues outside of the CDRs from the donor antibody
are not desired in the final grafted product.

78

Briefly, the protein sequence of the LM609 antibody, was determined by cloning and sequencing the cDNA that encodes the variable regions of the heavy and light chains as described in Example I. The CDRs from

5   the LM609 donor antibody were identified and grafted into homologous human variable regions of a human acceptor framework. Identification of CDR regions were based on the combination of definitions published by Kabat et al., and MacCallum et al.

10   The boundaries of the CDR regions have been cumulatively defined by the above two publications and are residues 30-35, 47-66 and 97-106 for CDRs 1, 2 and 3, respectively, of the heavy chain variable region and residues 24-36, 46-56, and 89-97 for CDRs 1, 2 and 3,

15 respectively, of the light chain variable region. Non-identical donor residues within these boundaries but outside of CDRs as defined by Kabat et al. were identified and were not substituted into the acceptor framework. Instead, functional non-donor amino acid

20 residues were identified and substituted for certain of these non-identical residues.

As described below, the only non-identical residue outside of the CDRs as defined by Kabat et al. but within the CDRs as defined above is at position 49 of

25 the LM609 light chain. To identify functional non-donor amino acids at this position, a library of nineteen antibodies was constructed that contained all non-donor amino acids at position 49 and then screened for binding activity against $\alpha v \beta_3$.

30   Human immunoglobulin sequences were identified from the Brookhaven Protein Data Bank-Kabat Sequences of Proteins of Immunological Interest database (release

79

5.0). Human framework sequences showing significant
identity to the murine LM609 variable region gene
sequences were selected for receiving the LM609 CDRs.
Human heavy chain variable region M72 `CL had 88%

5   identity to frameworks 1, 2 and 3 of LM609 heavy chain
and human light chain V region LS1 `CL had 79% identity
to frameworks 1, 2 and 3 of LM609 light chain. With the
exclusion of non-identical residues outside of the CDRs
as defined by Kabat et al. murine LM609 CDR sequences as

10   defined by Kabat et al. and MacCallum et al. were grafted
onto the human frameworks. Using this grafting scheme,
the final grafted product does not contain any amino acid
residues outside of the CDRs as defined by Kabat et al.
which are identical to an LM609 amino acid at the

15   corresponding position (outside of residues: 31-35, 50-66
and 99-106 for CDRs 1, 2 and 3, respectively, of the
heavy chain variable region and residues 24-34, 50-56,
and 89-97 for CDRs 1, 2 and 3, respectively, of the light
chain variable region). Moreover, no intermediates are

20   produced which contain an amino acid residue outside of
the CDRs as defined by Kabat et al. which are identical
to the LM609 amino acid at that position. The CDR
grafting procedures are set forth below.

        Full-length CDR grafted variable region genes

25   were synthesized by PCR using long overlapping
oligonucleotides as described previously in Example II.
The heavy chain variable region oligonucleotides were
those described previously as SEQ ID NOS:9-13. The light
chain variable region oligonucleotides were synthesized

30   so as to contain the CDR grafted variable region as well
as a stop codon at position 49. The five
oligonucleotides for the light chain LM609 grafted
variable region are show as SEQ ID NOS:23-27 where the
second oligonucleotide in the series contains the stop

80

codon at position 49 (SEQ ID NO:24). The nucleotide
sequences of oligonucleotides used to construct LM609
grafted light chain variable region is shown in Table 7.

Table 7: Oligonucleotides Used to Construct LM609

5   **Grafted Light Chain Variable Region**


```
    GAGATTGTGC TAACTCAGTC TCCAGCCACC CTGTCTCTCA GCCCAGGAGA AAGGGCGACT
    CTTTCCTGCC AGGCCAGCCA AAGTATT               SEQ ID NO: 23
    TTAGATGAGA AGCCTTGGGG CTTGACCAGG CCTTTGTTGA TACCAGTGTA GGTGGTTGCT
    AATACTTTGG CTGGC                            SEQ ID NO: 24
10  CCAAGGCTTC TCATCTAATA TCGTTCCCAG TCCATCTCTG GGATCCCCGC CACGTTCAGT
    GGCAGTGGAT CAGGGACAGA TTTC                  SEQ ID NO: 25
    GCTGCCACTC TGTTGACAGT AATAGACTGC AAAATCTTCA GGCTCCAGAC TGGAGATAGT
    GAGGGTGAAA TCTGTCCCTG A                     SEQ ID NO: 26
    CAACAGAGTG GCAGCTGGCC TCACACGTTC GGAGGGGGGA CCAAGGTGGA AATTAAG
15                                             SEQ ID NO:27
```


            All long oligonucleotides were gel purified.
    CDR grafting of the LM609 heavy chain variable region was
    constructed by mixing 5 overlapping oligonucleotides (SEQ
    ID NOS:9-13), at equimolar concentrations, in the
20 presence of annealing PCR primers containing at least 18
    nucleotide residues complementary to vector sequences for
    the efficient annealing of the amplified V region product
    to the single-stranded vector. The annealed mixture was
    fully converted to a double-stranded molecule with T4 DNA
25 polymerase plus dNTPs and ligated with T4 ligase. The
    mutagenesis reaction (1 µl) was electroporated into *E.
    coli* strain DH10B (BRL), titered onto a lawn of XL-1
    (Stratagene, Inc.) and incubated until plaques formed.
    Replica filter lifts were prepared and plaques containing
30 V_H gene sequences were screened either by hybridization
    with a digoxigenin-labeled oligonucleotide complementary
    to LM609 heavy chain CDR 2 sequences or reactivity with
    7F11-alkaline phosphatase conjugate, a monoclonal

81

antibody raised against the decapeptide sequence Tyr Pro
Tyr Asp Val Pro Asp Tyr Ala Ser (SEQ ID NO:28) appended
to the carboxy terminus of the vector CH_1 domain (Biosite,
Inc., San Diego, CA).

5   Fifty clones that were double-positive were
pooled and used to prepare uridinylated template for
hybridization mutagenesis with the amplified CDR grafted
LM609 V_L product constructed in a similar fashion using
the five overlapping oligonucleotides shown as SEQ ID
10   NOS:23-27. The mutagenesis reaction was electroporated
into *E. coli* strain DH10B. Randomly picked clones were
sequenced to identify a properly constructed template for
construction of the non-donor library at position 49.
This template was prepared as a uridinylated template and
15   an oligonucleotide population of the following sequence
was used for site directed mutagenesis.

               GGGAACGATA-19aa-GATGAGAAGC

The sequence 19aa in the above primer (SEQ ID NO:30)
represents the fact that this primer specifies a sequence
20   population consisting of 19 different codon sequences
that encode each of the 19 non-donor amino acids. These
amino acids are those not found at position 49 of LM609
and include all amino acids except for Lys. Clones that
resulted from this mutagenesis were picked and antibody
25   expressed by these clones were prepared. These samples
were then screened for binding to $\alpha v \beta_3$ in an ELISA assay.
Clones having either Arg or Met amino acids in position
49 were functionally identified. The nucleotide and
amino acid sequence of the LM609 grafted heavy chain
30   variable region is show in Figure 1A (SEQ ID NOS:1 and 2,
respectively). The nucleotide and amino acid sequence of

82

the LM609 grafted light chain variable region is shown in Figure 7 (SEQ ID NOS:31 and 32, respectively).

EXAMPLE VI

Generation of LM609 Grafted Antibodies Having Enhanced Activity

This example shows *in vitro* maturation of LM609 grafted antibody to obtain antibody variants having increased affinity to $\alpha_V\beta_3$ relative to the parent LM609 grafted antibody.

To optimize the affinity of LM609 grafted antibody *in vitro*, an M13 phage system was used, which permits the efficient synthesis, expression, and screening of libraries of functional antibody fragments (Fabs). The contribution of each of the six CDRs of the Ig heavy and light chains was assessed. The CDRs were defined broadly based on a combination of sequence variability and antibody structural models (Kabat et al., J. Biol. Chem. 252:6609-6616 (1977); Chothia et al., *supra*; MacCallum et al., *supra*). Thus, one library was constructed for each CDR, with the exception of H2 which was split into two libraries due to its long (20 amino acids) length. The variable region frameworks which harbored the mutated CDRs were the heavy chain variable region shown in Figure 1a (SEQ ID NO:2) and the light chain variable region shown in Figure 7 (SEQ ID NO:32).

CDRs were chosen from the heavy chain variable region shown in Figure 1a (SEQ ID NO:2) and the light chain variable region shown in Figure 7 (SEQ ID NO:32). Briefly, utilizing the numbering system of Kabat et al., *supra*, the residues chosen for mutagenesis of the CDRs

(Table 9) were: Gln^{24}-Tyr^{36} in light chain CDR1 (L1);
Leu^{46}-Ser^{56} in light chain CDR2 (L2); Gln^{89}-Thr^{97} in light
chain CDR3 (L3); Gly^{26}-Ser^{35} in heavy chain CDR1 (H1);
Trp^{47}-Gly^{65} in heavy chain CDR2 (H2); and Ala^{93}-Tyr^{102} in

5   heavy chain CDR3 (H3). Libraries were created for each
CDR, with the oligonucleotides designed to mutate a
single CDR residue in each clone. Due to the extended
length of H2, two libraries mutating residues 47-55 (H2a)
and 56-65 (H2b), respectively, were constructed to cover

10 this region.

     The template for generating light chain CDR3
mutants contained Gly at position 92. However, it was
subsequently determined that position 92 of the light
chain CDR3 was inadvertently deduced to be a Gly,

15 resulting in humanized LM609 grafted antibodies being
constructed with Gly at that position. It was later
realized that the original LM609 sequence contained an
Asn at position 92. Using the methods described herein
to introduce mutations into CDRs of an LM609 grafted

20 antibody, an LM609 grafted antibody having Asn at
position 92 of light chain CDR3 was found to have $\alpha_v\beta_3$
binding activity (see Table 9), confirming the
identification of Asn^{92} as a functional LM609 grafted
antibody. Thus, antibodies containing light chain CDR3

25 having Gly or Asn at position 92 are active in binding
$\alpha_v\beta_3$.

     Oligonucleotides encoding a single mutation
were synthesized by introducing NN(G/T) at each CDR
position as described previously (Glaser et al., *supra*).

30 The antibody libraries were constructed in M131XL604
vector by hybridization mutagenesis as described
previously, with some modifications (Rosok et al., J.
Biol. Chem. 271:22611-22618 (1996); Huse et al., J.

84

Immunol. 149:3914-3920 (1992); Kunkel, *Proc. Natl. Acad. Sci. USA* 82:488-492 (1985); Kunkel et al., *Methods Enzymol.* 154:367-382 (1987)). Briefly, the oligonucleotides were annealed at a 20:1 molar ratio to
5   uridinylated LM609 grafted antibody template (from which the corresponding CDR had been deleted) by denaturing at 85°C for 5 min, ramping to 55°C for 1 h, holding at 55°C for 5 min, then chilling on ice. The reaction was extended by polymerization electroporated into DH10B and
10   titered onto a lawn of XL-1 Blue. The libraries consisted of pools of variants, each clone containing a single amino acid alteration in one of the CDR positions. Utilizing codon-based mutagenesis, every position in all of the CDRs was mutated, one at a time, resulting in the
15   subsequent expression of all twenty amino acids at each CDR residue (Glaser et al., *supra*). The CDR libraries ranged in size from 288 (L3) to 416 (L1) unique members and contained a total of 2336 variants.

        To permit the efficient screening of the
20   initial libraries, a highly sensitive plaque lift assay, termed capture lift, was employed (Watkins et al., *Anal. Biochem.* 256 (1998)). Briefly, phage expression libraries expressing LM609 grafted antibody variants were initially screened by a modified plaque lift approach, in
25   which the nitrocellulose was pre-coated with goat anti-human kappa antibody and blocked with bovine serum albumin prior to application to the phage-infected bacterial lawn. Following the capture of phage-expressed LM609 grafted antibody variant Fabs, filters were
30   incubated with 1.0 μg/ml biotinylated $\alpha_v\beta_3$ for 3 h at 4°C, washed four times, incubated with 2.3 μg/ml NeutrAvidin-alkaline phosphatase (Pierce Chemical Co.; Rockford, IL) for 15 min at 25°C, and washed four times. All dilutions and washes were in binding buffer.

85

Variants that bound $\alpha_v\beta_3$ were identified by incubating the filters for 10-15 min in 0.1 M Tris, pH 9.5, containing 0.4 mM 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3, 3'-(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium

5   chloride and 0.38 mM 5-bromo-4-chloro-3-indoxyl phosphate mono-(p-toluidinium) salt (JBL Scientific, Inc.; San Luis Obispo, CA).

      To generate biotinylated $\alpha_v\beta_3$, the $\alpha_v\beta_3$ receptor was purified from human placenta by affinity

10   chromatography, as described previously (Smith and Cheresh, _J. Biol. Chem._ 263:18726-18731 (1988)). To biotinylate $\alpha_v\beta_3$, purified receptor was dialyzed into 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.0 mM $CaCl_2$, containing 0.1% NP-40 (binding buffer) and incubated with 100-fold

15   molar excess sulfosuccinimidobiotin for 3h at 4°C. The reaction was terminated by the addition of 50 mM ethanolamine.

      Phage expressed LM609 grafted antibody variants were selectively captured on nitrocellulose filters

20   coated with goat anti-human kappa chain antibody, probed with biotinylated $\alpha_v\beta_3$, and detected with NeutrAvidin-alkaline phosphatase. Initially, biotinylated $\alpha_v\beta_3$ was titrated on lifts containing phage expressing the LM609 grafted antibody parent molecule

25   only. Subsequently, the concentration of biotinylated $\alpha_v\beta_3$ was decreased to yield a barely perceptible signal. In this way, only clones expressing higher affinity variants were readily identified during screening of the variant libraries. Following the exhaustive capture lift

30   screening of ≥2500 clones from each library, 300 higher affinity variants were identified (see Table 10). The greatest number of clones displaying improved affinity were identified in the H3 (185) and L3 (52) CDRs, though

86

variants with improved affinity were identified in every
CDR.

        LM609 grafted antibody variants identified by
capture lift as having $\alpha_v\beta_3$ binding activity were further
5   characterized to determine binding affinity to $\alpha_v\beta_3$,
specificity for $\alpha_v\beta_3$ over other integrins, and $\alpha_v\beta_3$
association and dissociation rates. For these assays,
purified Fab of LM609 grafted antibody variants was used.
Briefly, Fab was expressed as described previously and
10   was released from the periplasmic space by sonic
oscillation (Watkins et al., *supra*, 1997). Cells
collected from one liter cultures were lysed in 10 ml
50 mM Tris, pH 8.0, containing 0.05% Tween 20. Fab was
bound to a 1 ml protein A column (Pharmacia) which had
15   been equilibrated with 50 mM glycine, pH 8, containing
250 mM NaCl, washed with the same buffer, and eluted with
10 ml of 100 mM glycine, pH 3, into one-tenth volume 1 M
Tris, pH 8. Purified Fab was quantitated as described
previously (Watkins et al., *supra*, 1997).

20   LM609 grafted antibody variants were tested for
binding to $\alpha_v\beta_3$ and specificity of binding to $\alpha_v\beta_3$ relative
to $\alpha_v\beta_5$ and $\alpha_{IIb}\beta_3$. For ELISA titration of Fab on
immobilized $\alpha_v\beta_3$ and the related integrins $\alpha_v\beta_5$ and $\alpha_{IIb}\beta_3$,
Immulon II microtiter plates were coated with 1 μg/ml
25   purified receptor in 20 mM Tris, pH 7.4, 150 mM NaCl, 2
mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, washed once, and blocked
in 3% BSA in 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$,
1 mM $MgCl_2$ for 1 h at 25°C. Human $\alpha_{IIb}\beta_3$, purified from
platelets, was obtained from Enzyme Research
30   Laboratories, Inc. (South Bend, IN) and $\alpha_v\beta_5$ was purified
from placental extract depleted of $\alpha_v\beta_3$, as described
previously (Smith et al., <u>J. Biol. Chem.</u> 265:11008-11013
(1990)). Just prior to use, the plates were washed two

87

times and were then incubated 1 h at 25°C with various
dilutions of Fab.   The plates were washed five times,
incubated 1 h at 25°C with goat anti-human kappa-alkaline
phosphatase diluted 2000-fold, washed five times, and
5   developed as described previously (Watkins et al., *supra*,
1997).   All dilutions and washes were in 50 mM Tris-HCl,
pH 7.4, 100 mM NaCl, 2 mM CaCl$_2$, and 1 mM MgCl$_2$.

Table 10:   Capture Lift Screening of LM609 grafted
antibody CDR Libraries.

	Library	Size[1]	Screened[2]	Positives[3]	Enhanced Affinity[4]
10	H1	320	2500	16	8
	H2a	320	5000	26	7
	H2b	320	5000	2	1
	H3	320	5000	185	78[5]
15	L1	416	2500	12	1
	L2	352	3250	7	1
	L3	288	5000	52	41

[1]Number of unique clones based on DNA sequence.   Thirty-
two condons are used to express all twenty amino acids at
20   each position.

[2]Phage-expressed libraries were plated on XL-1 Blue/agar
lawns at 500-100 plaques per 100 mm dish.

[3]Positives are defined as clones that were identified in
the initial screen, replated, and verified in a second
25   capture lift assay.

[4]Soluble Fab was titrated against immobilized $\alpha_v\beta_3$ in an
ELISA format.   Based on comparison of the inflection

88

point of the titration profiles, clones which displayed
~3-fold enhanced affinity were selected for further
characterization.

[5]Of the 185 positive clones identified by capture lift, 98
5   were further characterized for binding to immobilized
$\alpha_V\beta_3$.

Figure 8 shows titration of antibody variants
and LM609 grafted antibody Fab on immobilized $\alpha_V\beta_3$.
Bacterial cell lysates containing LM609 grafted antibody
10 (closed circles), variants with improved affinity
isolated from the primary libraries (S102, closed
squares; Y100, open squares; and Y101, open triangles) or
from the combinatorial libraries (closed triangles), or
an irrelevant Fab (open circles) were titrated on
15 immobilized $\alpha_V\beta_3$.

Comparison of the inflection points of the
binding profiles obtained from titrating variants on
immobilized $\alpha_V\beta_3$ demonstrated that multiple clones
displayed >3-fold improved affinity, confirming the
20 effectiveness of utilizing the capture lift in a
semi-quantitative fashion (Figure 8, compare squares and
open triangles with closed circles). Based on the
capture lift screening and subsequent characterization of
binding to immobilized $\alpha_V\beta_3$, it was concluded that both
25 heavy and light chain CDRs are directly involved in the
interaction of $\alpha_V\beta_3$ with the LM609 grafted antibody
variants.

DNA was isolated from clones displaying >3-fold
enhanced binding and sequenced to identify the mutations
30 which resulted in higher affinity. DNA sequencing was
performed on isolated single-stranded DNA. The heavy and
light chain variable region genes were sequenced by the

89

fluorescent dideoxynucleotide termination method
(Perkin-Elmer; Foster City, CA). Based on sequence
analysis of 103 variants, 23 unique mutations clustered
at 14 sites were identified (Table 9). The majority of
5   the sites of beneficial mutations were found in the heavy
chain CDRs, with four located in H3, and three each in H2
(2a and 2b combined) and H1. Seven distinct and
beneficial amino acid substitutions were identified at a
single site within H3, tyrosine residue 102. The diverse
10   nature of the substitutions at this site suggests that
tyrosine residue 102 may sterically hinder LM609 grafted
antibody binding to $\alpha_v\beta_3$. In support of this, variants
expressing the other aromatic amino acids (phenylalanine,
histidine, and tryptophan) instead of tyrosine at residue
15   102 were never isolated following screening for enhanced
binding.

The affinities of select variants were further
characterized by utilizing surface plasmon resonance
(BIAcore) to measure the association and dissociation
20   rates of purified Fab with immobilized $\alpha_v\beta_3$. Briefly,
surface plasmon resonance (BIAcore; Pharmacia) was used
to determine the kinetic constants for the interaction
between $\alpha_v\beta_3$ and LM609 grafted antibody variants.
Purified $\alpha_v\beta_3$ receptor was immobilized to a
25   (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
hydrochloride)/N-hydroxysuccinimide-activated sensor chip
by injecting 30 μl of 15 μg/ml $\alpha_v\beta_3$ in 10 mM sodium
acetate, pH 4. To obtain association rate constants
(k_{on}), the binding rate at five different Fab
30   concentrations, ranging from 5-40 μg/ml in 50 mM
Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$,
was determined at a flow rate of 10 μl/min. Dissociation
rate constants (k_{off}) were the average of five
measurements obtained by analyzing the dissociation phase

90

at an increased flow rate (40 μl/min). Sensorgrams were
analyzed with the BIAevaluation 2.1 program (Pharmacia).
Residual Fab was removed after each measurement with
10 mM HCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$.

5   Table 9 shows that the variants all displayed a
lower Kd than the LM609 grafted antibody parent molecule,
consistent with both the capture lift and the ELlSA.
Analysis of association and dissociation rates revealed
that the majority of improved variants had slower
10   dissociation rates while having similar association
rates. For example, LM609 grafted antibody had an
association rate 18.0 x 10^4 $M^{-1}s^{-1}$, while the variants
ranged from 16.7-31.8 x 10^4 $M^{-1}s^{-1}$. In contrast, every
clone dissociated slower than LM609 grafted antibody
15   (4.97 x 10^{-3} s $^{-1}$) with dissociation rates ranging from
1.6-fold (3.03 x 10^{-3} s^{-1}) to 11.8-fold (0.42 x 10^{-3} s^{-1})
slower.

            These results demonstrate that introducing
single amino acid substitutions into LM609 grafted
20   antibody CDRs allows the identification of modified LM609
grafted antibodies having higher affinity for $\alpha_v\beta_3$ than
the parent LM609 grafted antibody.

91

Table 9: Identification of Enhanced LM609 Grafted Antibodies from Primary Libraries

chain*	library†	sequence	k_{on} (x10^4) ($M^{-1}s^{-1}$)	k_{off} (x10^-3) (s^{-1})	Kd (nM)
	LM609 grafted antibody		18.0	4.97	27.6
H	CDR1 T27 W29 L30	G F T F S S Y D M S **T** **W** **L**	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.
H	CDR2a K52	W V A K V S S G G G **K**	17.8	2.18	12.2
H	CDR2b P60 E64	S T Y Y L D T V Q G **P** **E**	31.8 n.d.	1.85 n.d.	5.8 n.d.
H	CDR3 H97 Y100 D101 Y101 S102 T102 D102 E102 M102 G102 A102	A R H N Y G S F A Y **H** **Y** **D** **Y** **S** **T** **D** **E** **M** **G** **A**	22.0 17.5 n.d. 21.8 24.2 24.6 27.6 n.d. n.d. 16.1 27.5	3.03 2.51 n.d. 0.48 1.44 1.43 0.97 n.d. n.d. 2.01 2.27	13.8 14.3 n.d. 2.2 6.0 5.8 3.5 n.d. n.d. 12.5 8.3
L	CDR1 F32	Q A S Q S I S N H L H W Y **F**	16.7	0.42	2.5
L	CDR2 S51	L L I R Y R S Q S I S **S**	n.d.	n.d.	n.d.
L	CDR3 N92 T92 L96 Q96	Q Q S G S W P H T **N** **T** **L** **Q**	23.6 n.d. 24.3 n.d.	1.35 n.d. 2.23 n.d.	5.7 n.d. 9.2 n.d.

5

92

EXAMPLE VII

Generation of High Affinity LM609 Grafted Antibodies

       This example shows that single amino acid
mutations in CDRs of an LM609 grafted that result in
5   higher affinity binding to $\alpha_v\beta_3$ can be combined to
generate high affinity LM609 grafted antibodies.

       Random combination of all of the beneficial
mutations of LM609 grafted antibody would generate a
combinatorial library containing >10^5 variants, requiring
10   efficient screening methodologies. Therefore, to
determine if clones displaying >10-fold enhanced
affinities could be rapidly distinguished from one
another, variants displaying 3 to 13-fold enhanced
affinity were evaluated by capture lift utilizing lower
15   concentrations of biotinylated $\alpha_v\beta_3$. Despite repeated
attempts with a broad range of concentrations of $\alpha_v\beta_3$,
consistent differences in the capture lift signals were
not observed. Because of this, smaller combinatorial
libraries were constructed and subsequently screened by
20   EL1SA.

       Four distinct combinatorial libraries were
constructed in order to evaluate the optimal number of
combinations that could be accomplished utilizing two
site hybridization mutagenesis (Figure 9). Briefly,
25   combinatorial libraries were constructed by synthesizing
degenerate oligonucleotides encoding both the wild-type
and beneficial heavy chain mutations (H2, Leu[60]→Pro; H3
Tyr[97]→His; H3, Ala[101] →Tyr; H3, Tyr[102]→Ser, Thr, Asp, Glu,
Met, Gly, Ala). Utilizing two site hybridization
30   mutagenesis, as described above, the oligonucleotides
were annealed at a 40:1 molar ratio to uridinylated
template prepared from LM609 grafted antibody and three

93

light chain mutations (Figure 9; L1, His^{32}→Phe; L3,
Gly^{92}→Asn; L3, His^{96}→Leu). As a result, a total of 256
variants were synthesized in four combinatorial library
subsets.

5   Figure 9 shows construction of combinatorial
libraries of beneficial mutations. Uridinylated template
from LM609 grafted antibody and three optimal light chain
variants (F32, N92, and L96) was prepared. Two site
hybridization was performed with two degenerate
10   oligonucleotides, which were designed to introduce
beneficial mutations at four distinct heavy chain
residues.

          Following preparation of uridinylated templates
of LM609 grafted antibody and three light chain variants,
15   (Table 9; F32, N92, and L96), degenerate oligonucleotides
encoding the wild type residue and the most beneficial
heavy chain mutations (Table 9; P60, H97, Y101, S102,
T102, D102, E102, M102, G102, and A102) were hybridized
to the light chain templates, resulting in four
20   combinatorial libraries, each containing 64 unique
variants. Potentially, the combination of multiple
mutations can have detrimental effects on affinity and,
thus, can prevent the identification of beneficial
combinations resulting from mutations at fewer sites.
25   For this reason, the amino acid expressed by the LM609
grafted antibody parent molecule was included at each
position in the combinatorial library. By utilizing this
approach, simultaneous combinatorial mutagenesis of three
CDRs (L1 or L3 each in combination with H2 and H3) was
30   accomplished. Based on sequence analysis, the two site
hybridization mutagenesis was achieved with ~50%
efficiency.

94

In order to screen the combinatorial libraries, soluble Fab was expressed and released from the periplasm of small-scale (<1 ml) bacterial cultures that had been infected with randomly selected clones. Although

5   variable expression levels were observed, uniform quantities of the unpurified variants were captured on a microtiter plate through a peptide tag present on the carboxyl-terminus of the heavy chain. Briefly, combinatorial LM609 grafted antibody libraries were

10   screened by an EL1SA that permits the determination of relative affinities of antibody variants produced in small-scale bacterial cultures (Watkins et al., <u>Anal. Biochem.</u> 253:37-45 (1997)). An Immulon II microtiter plate (Dynatech Laboratories; Chantilly, VA) was coated

15   with 10 μg/ml of the 7F11 monoclonal antibody, which recognizes a peptide tag on the carboxyl-terminus of the LM609 grafted antibody variant heavy chains (Field et al., <u>Mol. Cell. Biol.</u> 8:2159-2165 (1988)). Following capture of Fab from *E. coli* lysates, the plate was

20   incubated with 0.5-1 μg/ml biotinylated $\alpha_V\beta_3$ for 1 h at 25°C. The plate was washed seven times, incubated with 0.5 U/ml streptavidin-alkaline phosphatase (1000 U/ml; Boehringer Mannheim; Indianapolis, IN) for 15 min at 25°C, washed seven times, and developed as described

25   previously (Watkins et al., *supra*, 1997). All dilutions and washes were in binding buffer.

As described previously (Watkins et al., *supra*, 1997), this ELISA screening method enabled a rapid and direct comparison of the relative affinities of the

30   variants following incubation with biotinylated $\alpha_V\beta_3$ and streptavidin-alkaline phosphatase. To ensure that the full Fab diversity was sampled, one thousand randomly selected clones were screened from each combinatorial library. Variants that displayed an enhanced EL1SA

95

signal were further characterized for binding to
immobilized $\alpha_v\beta_3$ (Figure 8, closed triangles) and were
sequenced to identify the mutations (Table 10).

Screening of the four combinatorial libraries
5   identified fourteen unique combinations of mutations that
improved binding significantly over the individual
mutations identified in the screening of the first
library. While the best clone from the primary screen
had a 12.5-fold increase in affinity, the fourteen unique
10   combinations isolated from screening the combinatorial
libraries displayed affinities ranging from 18 to 92-fold
greater than the parent LM609 grafted antibody. The
majority of these variants consisted of H2 and H3
mutations combined with the L1 or L3 mutations.
15   Beneficial combinations of heavy chain mutations with
wild-type light chain were also identified, but did not
result in improved affinity to the same extent as other
combinatorial variants. The variants predominantly
contained 2 to 4 mutations, with one clone, C29,
20   containing five mutations. No direct correlation between
the total number of mutations in each variant and the
resulting affinity was observed. For example, while the
binding of clone C37 was 92-fold enhanced over the parent
molecule and was achieved through the combination of
25   three mutations, clone C29 had ~55-fold greater affinity
achieved through the combination of five mutations.
Multiple variants displaying >50-fold enhanced affinity
resulting from the combination of as few as two mutations
were identified (2G4, 17, and V357D).

30   The combinatorial clones with improved affinity
all displayed >10-fold slower dissociation rates,
possibly reflecting a selection bias introduced by long
incubation steps in the screening. In addition, all of

96

the combinatorial variants isolated from the library
based on the L96 light chain mutation also displayed 2 to
4-fold greater association rates. Previously, it has
been demonstrated that the antibody repertoire shifts
5   towards immunoglobulins displaying higher association
rates during affinity maturation *in vivo* (Foote and
Milstein, Nature 352:530-532 (1991)). The L96 subset of
variants, therefore, may more closely mimick the *in vivo*
affinity maturation process where B-lymphocyte
10   proliferation is subject to a kinetic selection.

        LM609 grafted antibody binds the $\alpha_V\beta_3$ complex
specifically and does not recognize either the α_V or the
β_3 chain separately. To further characterize the
variants, clones were screened for reactivity with the
15   related integrins, $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_5$. All variants tested
were unreactive with both $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_5$, consistent with
the improved binding not substantially altering the
interaction of Fab and receptor.

Table 10: Identification of Optimal Combinatorial Mutations

library*	clone	sequence†							k_{on} (x10⁴) (M⁻¹·s⁻¹)	k_{on} (x10⁻³) (s⁻¹)	Kd (nM)
		L1 32	L3 92	L3 96	H2 60	H3 97	H3 101	H3 102			
wild type		H	G	H	L	Y	A	Y	18.0	4.97	27.6
F32	17	F						S	25.1	0.138	0.5
	7	F			P	H		S	20.4	0.236	1.2
	56	F			P			S	26.6	0.135	0.5
	C59	F			P			D	26.5	0.137	0.5
	C176	F			P			T	22.5	0.192	0.9
	V357D	F						D	27.9	0.140	0.5
N92	C119		N		P			S	21.5	0.316	1.5
L96	8F9			L	P	H		S	47.5	0.280	0.6
	C29			L	P	H	Y	S	67.5	0.343	0.5
	2G4			L				S	60.3	0.229	0.4
	6H6			L		H		S	50.4	0.187	0.4
	C37			L			Y	E	44.8	0.147	0.3
	6D1			L	P		Y	S	41.0	0.158	0.4
	6G1			L	P			S	38.9	0.280	0.7

98

As a first step toward determining if the increase in affinity of the variants resulted in greater biological activity, variants displaying a range of affinities were assayed for their ability to inhibit the
5   binding of a natural ligand, fibrinogen, to immobilized $\alpha_v\beta_3$ receptor. Briefly, LM609 grafted antibody variants were tested for inhibition of ligand binding as described previously except that the binding of biotinylated human fibrinogen (Calbiochem, La Jolla, CA) was detected with
10   0.5 μg/ml NeutrAvidin-alkaline phosphatase (Smith et al., J. Biol. Chem. 265:12267-12271 (1990)).

The results of these competition assays are shown in Figure 10. Figure 10A shows inhibition of fibrinogen binding to immobilized $\alpha_v\beta_3$. Immobilized $\alpha_v\beta_3$
15   was incubated with 0.1 μg/ml biotinylated fibrinogen and various concentrations of LM609 grafted antibody (open circles), S102 (closed circles), F32 (open triangles), or C59 (closed triangles) for 3 h at 37°C. Unbound ligand and Fab were removed by washing and bound fibrinogen was
20   quantitated following incubation with NeutrAvidin alkaline phosphatase conjugate. Figure 10B shows correlation of affinity of variants with inhibition of fibrinogen binding. The concentration of variants required to inhibit the binding of fibrinogen to
25   immobilized $\alpha_v\beta_3$ by 50% (IC_{50}) was plotted as a function of the affinity (Kd).

As shown in Figure 10A, higher affinity variants were more effective at blocking the ligand binding site of the receptor (compare LM609 grafted
30   antibody, open circles, with any of the variants). Subsequent analysis of ten variants displaying affinities (Kd) ranging from 0.3 to 27 nm demonstrated a good correlation (r^2 = 0.976) between affinity and ability to

99

inhibit fibrinogen binding (Figure 10B). In addition,
the variants were tested for inhibition of vitronectin
binding to the receptor. Similar to fibrinogen, the
variants were more effective at inhibiting the
5   interaction than the parent molecule. Thus, consistent
with the cross-reactivity studies with related integrin
receptors, mutations which increased affinity did not
appear to substantially alter the manner in which the
antibody interacted with the receptor.

10   The ability of the variants to inhibit the
adhesion of M21 human melanoma cells expressing the $\alpha_v\beta_3$
receptor to fibrinogen was examined. Inhibition of the
adhesion of 4×10^4 M21 cells to fibrinogen by the LM609
grafted antibody variants was performed as described
15   previously (Leavesley et al., J. Cell Biol. 117:1101-1107
(1992)). Similar to the ligand competition studies with
purified fibrinogen and $\alpha_v\beta_3$ receptor, higher affinity
variants were generally more effective at preventing cell
adhesion than was LM609 grafted antibody (Figure 11).
20   Figure 11 shows inhibition of M21 human melanoma cell
adhesion to fibrinogen. Cells and various concentrations
of LM609 grafted antibody Fab (closed triangles), S102
(open circles), G102 (closed circles), or C37 (open
triangles) were added to 96 well cell culture plates
25   which had been coated with 10 μg/ml fibrinogen. After
incubating for 35 min at 37°C, unbound cells were removed
by washing and adherent cells were quantitated by crystal
violet staining.

Although intact LM609 grafted antibody Ig
30   inhibits cell adhesion, the phage expressed Fab did not
affect cell adhesion at concentrations as high as 1 mg/ml
(Figure 11, closed triangles). Clone C37, isolated from
the combinatorial library and displaying ~90-fold greater

affinity than LM609 grafted antibody Fab, inhibited cell
adhesion completely (Figure 11, open triangles). Variant
G102 had a moderately higher affinity (2.2-fold enhanced)
and also inhibited cell adhesion, though less effectively
5   than C37 (Figure 11, closed circles). Surprisingly,
clone S102 (Figure 11, open circles), which had a
4.6-fold higher affinity than LM609 grafted antibody, was
ineffective at inhibiting cell adhesion, suggesting that
clones G102 and S102 interact with the $\alpha_v\beta_3$ receptor
10   differently.

      These results show that combining single amino
acid mutations that result in LM609 grafted antibodies
exhibiting higher binding affinity to $\alpha_v\beta_3$ allows the
identification of high affinity LM609 grafted antibody
15   mutants having greater than 90-fold higher binding
affinity than the parent LM609 grafted antibody.

      Although the invention has been described with
reference to the disclosed embodiments, those skilled in
the art will readily appreciate that the specific
20   experiments detailed are only illustrative of the
invention. It should be understood that various
modifications can be made without departing from the
spirit of the invention. Accordingly, the invention is
limited only by the following claims.

101

What is claimed is:

1.   A Vitaxin antibody exhibiting selective binding affinity to $\alpha_v\beta_3$ comprising at least one Vitaxin heavy chain polypeptide comprising substantially the same variable region amino acid sequence as that shown in Figure 1A (SEQ ID NO:2) and at least one Vitaxin light chain polypeptide comprising substantially the same variable region amino acid sequence as that shown in Figure 1B (SEQ ID NO:4) or a functional fragment thereof.

2.   The Vitaxin antibody of claim 1, wherein said functional fragment is selected from the group consisting of Fv, Fab, F(ab)$_2$ and scFV.

3.   A nucleic acid encoding a Vitaxin heavy chain polypeptide comprising substantially the same Vitaxin heavy chain variable region nucleotide sequences as that shown in Figure 1A (SEQ ID NO:1) or a fragment thereof.

4.   The nucleic acid of claim 3, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as the variable region of said Vitaxin heavy chain polypeptide (SEQ ID NO:1).

5.   The nucleic acid of claim 3, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as a CDR of said Vitaxin heavy chain polypeptide.

6.   A nucleic acid encoding a Vitaxin light chain polypeptide comprising substantially the same Vitaxin light chain variable region nucleotide sequences as that shown in Figure 1B (SEQ ID NO:3) or a fragment
5   thereof.

7.   The nucleic acid of claim 6, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as the variable region of said Vitaxin light chain polypeptide
10   (SEQ ID NO:3).

8.   The nucleic acid of claim 6, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as a CDR of said Vitaxin light chain polypeptide.

15   9.   A nucleic acid encoding a Vitaxin heavy chain polypeptide comprising a nucleotide sequence encoding substantially the same Vitaxin heavy chain variable region amino acid sequence as that shown in Figure 1A (SEQ ID NO:2) or fragment thereof.

20   10.   The nucleic acid of claim 9, wherein said fragment further comprises a nucleic acid encoding substantially the same heavy chain variable region amino acid sequence of said Vitaxin heavy chain amino acid sequence (SEQ ID NO:2).

25   11.   The nucleic acid of claim 9, wherein said fragment further comprises a nucleic acid encoding substantially the same heavy chain CDR amino acid sequence of said Vitaxin heavy chain amino acid sequence.

    12.   A nucleic acid encoding a Vitaxin light
chain polypeptide comprising a nucleotide sequence
encoding substantially the same Vitaxin light chain
variable region amino acid sequence as that shown in
5   Figure 1B (SEQ ID NO:4) or fragment thereof.

    13.   The nucleic acid of claim 12, wherein said
fragment further comprises a nucleic acid encoding
substantially the same light chain variable region amino
acid sequence of said Vitaxin light chain amino acid
10   sequence (SEQ ID NO:4).

    14.   The nucleic acid of claim 12, wherein said
fragment further comprises a nucleic acid encoding
substantially the same light chain CDR amino acid
sequence of said Vitaxin light chain amino acid sequence.

15   15.   A Vitaxin heavy chain polypeptide
comprising substantially the same variable region amino
acid sequence as that shown in Figure 1A (SEQ ID NO:2) or
functional fragment thereof.

    16.   The Vitaxin heavy chain polypeptide of
20   claim 15, wherein said functional fragment comprises a
variable chain polypeptide or a CDR polypeptide.

    17.   A Vitaxin light chain polypeptide
comprising substantially the same variable region amino
acid sequence as that shown in Figure 1B (SEQ ID NO:4) or
25   a functional fragment thereof.

    18.   The Vitaxin light chain polypeptide of
claim 17, wherein said functional fragment comprises a
variable chain polypeptide or a CDR polypeptide.

104

19. A LM609 grafted antibody exhibiting
selective binding affinity to $\alpha_v\beta_3$ comprising at least one
LM609 grafted heavy chain polypeptide comprising
substantially the same variable region amino acid
5   sequence as that shown in Figure 1A (SEQ ID NO:2) and at
least one LM609 grafted light chain polypeptide
comprising substantially the same variable region amino
acid sequence as that shown in Figure 7 (SEQ ID NO:32) or
a functional fragment thereof.

10   20. The LM609 grafted antibody of claim 19,
wherein said functional fragment is selected from the
group consisting of Fv, Fab, F(ab)$_2$ and scFV.

21. A nucleic acid encoding a LM609 grafted
heavy chain polypeptide comprising substantially the same
15   LM609 grafted heavy chain variable region nucleotide
sequences as that shown in Figure 1A (SEQ ID NO:1) or a
fragment thereof.

22. The nucleic acid of claim 21, wherein said
fragment further comprises a nucleic acid encoding
20   substantially the same nucleotide sequence as the
variable region of said LM609 grafted heavy chain
polypeptide (SEQ ID NO:1).

23. The nucleic acid of claim 21, wherein said
fragment further comprises a nucleic acid encoding
25   substantially the same nucleotide sequence as a CDR of
said LM609 grafted heavy chain polypeptide.

24. A nucleic acid encoding a LM609 grafted light chain polypeptide comprising substantially the same LM609 grafted light chain variable region nucleotide sequences as that shown in Figure 7 (SEQ ID NO:31) or a

5 fragment thereof.

25. The nucleic acid of claim 24, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as the variable region of said LM609 grafted light chain

10 polypeptide (SEQ ID NO:31).

26. The nucleic acid of claim 24, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as a CDR of said LM609 grafted light chain polypeptide.

15 27. A nucleic acid encoding a LM609 grafted antibody heavy chain polypeptide comprising a nucleotide sequence encoding substantially the same LM609 grafted heavy chain variable region amino acid sequence as that shown in Figure 1A (SEQ ID NO:2) or fragment thereof.

20 28. The nucleic acid of claim 27, wherein said fragment further comprises a nucleic acid encoding substantially the same heavy chain variable region amino acid sequence of said LM609 grafted heavy chain amino acid sequence (SEQ ID NO:2).

25 29. The nucleic acid of claim 27, wherein said fragment further comprises a nucleic acid encoding substantially the same heavy chain CDR amino acid sequence of said LM609 grafted heavy chain amino acid sequence.

30. A nucleic acid encoding a LM609 grafted antibody light chain polypeptide comprising a nucleotide sequence encoding substantially the same LM609 grafted light chain variable region amino acid sequence as that

5 shown in Figure 7 (SEQ ID NO:32) or fragment thereof.

31. The nucleic acid of claim 30, wherein said fragment further comprises a nucleic acid encoding substantially the same light chain variable region amino acid sequence of said Vitaxin light chain amino acid

10 sequence (SEQ ID NO:32).

32. The nucleic acid of claim 30, wherein said fragment further comprises a nucleic acid encoding substantially the same light chain CDR amino acid sequence of said Vitaxin light chain amino acid sequence.

15 33. A LM609 grafted heavy chain polypeptide comprising substantially the same variable region amino acid sequence as that shown in Figure 1A (SEQ ID NO:2) or functional fragment thereof.

34. The LM609 grafted heavy chain polypeptide

20 of claim 33, wherein said functional fragment comprises a variable chain polypeptide or a CDR polypeptide.

35. A LM609 grafted light chain polypeptide comprising substantially the same variable region amino acid sequence as that shown in Figure 7 (SEQ ID NO:32) or

25 a functional fragment thereof.

36. The LM609 grafted light chain polypeptide of claim 35, wherein said functional fragment comprises a variable chain polypeptide or a CDR polypeptide.

107

37. A nucleic acid encoding a heavy chain polypeptide for monoclonal antibody LM609 comprising substantially the same heavy chain variable region nucleotide sequence as that shown in Figure 2A (SEQ ID

5   NO:5) or a fragment thereof.

38. The nucleic acid of claim 37, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as the variable region of said heavy chain polypeptide (SEQ ID

10   NO:5).

39. The nucleic acid of claim 37, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as a CDR of said heavy chain polypeptide.

15   40. A nucleic acid encoding a light chain polypeptide for monoclonal antibody LM609 comprising substantially the same light chain variable region nucleotide sequence as that shown in Figure 2B (SEQ ID NO:7) or a fragment thereof.

20   41. The nucleic acid of claim 40, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as the variable region of said light chain polypeptide (SEQ ID NO:7).

25   42. The nucleic acid of claim 40, wherein said fragment further comprises a nucleic acid encoding substantially the same nucleotide sequence as a CDR of said light chain polypeptide.

108

43. A nucleic acid encoding a heavy chain polypeptide for monoclonal antibody LM609 comprising a nucleotide sequence encoding substantially the same heavy chain variable domain amino acid sequence of monoclonal

5   antibody LM609 as that shown in Figure 2A (SEQ ID NO:6) or fragment thereof.

44. The nucleic acid of claim 43, wherein said fragment further comprises a nucleic acid encoding substantially the same heavy chain variable region amino

10 acid sequence of said monoclonal antibody LM609 (SEQ ID NO:6).

45. The nucleic acid of claim 43, wherein said fragment further comprises a nucleic acid encoding substantially the same heavy chain CDR amino acid

15 sequence as said monoclonal antibody LM609.

46. A nucleic acid encoding a heavy chain polypeptide for monoclonal antibody LM609 comprising a nucleotide sequence encoding substantially the same light chain amino acid sequence of monoclonal antibody LM609 as

20 that shown in Figure 2B (SEQ ID NO:8) or fragment thereof.

47. The nucleic acid of claim 46, wherein said fragment further comprises a nucleic acid encoding substantially the same light chain variable region amino

25 acid sequence of said monoclonal antibody LM609 (SEQ ID NO:8).

48. The nucleic acid of claim 46, wherein said fragment further comprises a nucleic acid encoding substantially the same light chain CDR amino acid

30 sequence as said monoclonal antibody LM609.

109

49. A method of inhibiting a function of $\alpha_v\beta_3$ comprising contacting $\alpha_v\beta_3$ with Vitaxin or a functional fragment thereof under conditions which allow binding of Vitaxin to $\alpha_v\beta_3$.

5   50. The method of claim 49, wherein said functional fragment is selected from the group consisting of Fv, Fab, F(ab)$_2$ and scFV.

51. The method of claim 49, wherein said function of $\alpha_v\beta_3$ is binding of $\alpha_v\beta_3$ to a ligand.

10   52. The method of claim 49, wherein said function of $\alpha_v\beta_3$ is integrin mediated signal transduction.

53. A method of treating an $\alpha_v\beta_3$-mediated disease comprising administering an effective amount of Vitaxin or a functional fragment thereof under conditions

15 which allow binding to $\alpha_v\beta_3$.

54. The method of claim 53, wherein said functional fragment is selected from the group consisting of Fv, Fab, F(ab)$_2$ and scFV.

55. The method of claim 53, wherein said

20 $\alpha_v\beta_3$-mediated disease is angiogenesis or restenosis.

56. An enhanced LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$, or a functional fragment thereof, comprising at least one amino acid substitution in one or more CDRs of a LM609

25 grafted heavy chain variable region polypeptide or a LM609 grafted light chain variable region polypeptide, wherein the $\alpha_v\beta_3$ binding affinity of said enhanced LM609 grafted antibody is maintained.

110

57.   The enhanced LM609 grafted antibody of
claim 56, wherein said $\alpha_V\beta_3$ binding affinity of said LM609
grafted antibody is enhanced.

58.   The enhanced LM609 grafted antibody of
5   claim 56, wherein said functional fragment is selected
from the group consisting of Fv, Fab, F(ab)$_2$ and scFV.

59.   The enhanced LM609 grafted antibody of
claim 56, wherein said CDR having at least one amino acid
substitution is selected from the group consisting of
10   V_H CDR1, V_H CDR2, V_H CDR3, V_L CDR1, V_L CDR2 and V_L CDR3.

60.   The enhanced LM609 grafted antibody of
claim 59, wherein said V_H CDR1 is selected from the group
consisting of the CDRs referenced as SEQ ID NO:48, SEQ ID
NO:50 and SEQ ID NO:52.

15   61.   The enhanced LM609 grafted antibody of
claim 59, wherein said V_H CDR2 is selected from the group
consisting of the CDRs referenced as SEQ ID NO:54, SEQ ID
NO:56 and SEQ ID NO:58.

62.   The enhanced LM609 grafted antibody of
20   claim 59, wherein said V_H CDR3 is selected from the group
consisting of the CDRs referenced as SEQ ID NO:60, SEQ ID
NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID
NO:70, SEQ ID NO:72, SEQ ID NO:74, SEQ ID NO:76, SEQ ID
NO:78, SEQ ID NO:80, SEQ ID NO:94, SEQ ID NO:96; SEQ ID
25   NO:98 and SEQ ID NO:100.

63.   The enhanced LM609 grafted antibody of
claim 59, wherein said V_L CDR1 is the CDR referenced as
SEQ ID NO:82.

64.   The enhanced LM609 grafted antibody of claim 59, wherein said V$_L$ CDR2 is the CDR referenced as SEQ ID NO:84.

65.   The enhanced LM609 grafted antibody of claim 59, wherein said V$_L$ CDR3 is selected from the group consisting of the CDRs referenced as SEQ ID NO:86, SEQ ID NO:88, SEQ ID NO:90 and SEQ ID NO:92.

66.   The enhanced LM609 grafted antibody of claim 56, wherein said enhanced LM609 grafted antibody comprises at least one amino acid substitution in two or more CDRs of a LM609 grafted heavy chain variable region polypeptide or a LM609 grafted light chain variable region polypeptide.

67.   The enhanced LM609 grafted antibody of claims 66, wherein said functional fragment is selected from the group consisting of Fv, Fab, F(ab)$_2$ and scFV.

68.   The enhanced LM609 grafted antibody of claim 66, wherein said CDR having at least one amino acid substitution is selected from the group consisting of V$_H$ CDR1, V$_H$ CDR2, V$_H$ CDR3, V$_L$ CDR1, V$_L$ CDR2 and V$_L$ CDR3.

69. The enhanced LM609 grafted antibody of claim 68, wherein said enhanced LM609 grafted antibody comprises the combination of CDRs selected from the group consisting of:

5   the V_L CDR1 referenced as SEQ ID NO:82 and the V_H CDR3 referenced as SEQ ID NO:68;

the V_L CDR1 referenced as SEQ ID NO:82, the V_H CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced as SEQ ID NO:68;

10   the V_L CDR1 referenced as SEQ ID NO:82, the V_H CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced as SEQ ID NO:72;

the V_L CDR1 referenced as SEQ ID NO:82, the V_H CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced

15 as SEQ ID NO:70;

the V_L CDR1 referenced as SEQ ID NO:82 and the V_H CDR3 referenced as SEQ ID NO:72;

the V_L CDR3 referenced as SEQ ID NO:86, the V_H CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced

20 as SEQ ID NO:68;

the V_L CDR3 referenced as SEQ ID NO:90 and V_H CDR3 referenced as SEQ ID NO:68; and

the V_L CDR3 referenced as SEQ ID NO:90, the V_H CDR2 referenced as SEQ ID NO:56 and V_H CDR3 referenced as

25 SEQ ID NO:68.

70. The enhanced LM609 grafted antibody of claim 66, wherein at least one of said CDRs has two or more amino acid substitutions.

71. The enhanced LM609 grafted antibody of

30 claims 70, wherein said functional fragment is selected from the group consisting of Fv, Fab, $F(ab)_2$ and scFV.

113

72. The enhanced LM609 grafted antibody of claim 70, wherein said CDR having at least one amino acid substitution is selected from the group consisting of V_H CDR1, V_H CDR2, V_H CDR3, V_L CDR1, V_L CDR2 and V_L CDR3.

5   73. The enhanced LM609 grafted antibody of claim 72, wherein said enhanced LM609 grafted antibody comprises the combination of CDRs selected from the group consisting of:

        the V_L CDR1 referenced as SEQ ID NO:82, the V_H
10 CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced as SEQ ID NO:94;
        the V_L CDR3 referenced as SEQ ID NO:90, the V_H CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced as SEQ ID NO:94;
15   the V_L CDR3 referenced as SEQ ID NO:90, the V_H CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced as SEQ ID NO:96;
        the V_L CDR3 referenced as SEQ ID NO:90 and the V_H CDR3 referenced as SEQ ID NO:94;
20   the V_L CDR3 referenced as SEQ ID NO:90 and the V_H CDR3 referenced as SEQ ID NO:98; and
        the V_L CDR3 referenced as SEQ ID NO:90, the V_H CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced as SEQ ID NO:100.

25   74. A high affinity LM609 grafted antibody exhibiting selective binding affinity to $\alpha_v\beta_3$, or a functional fragment thereof, comprising at least one amino acid substitution in one or more CDRs of a LM609 grafted heavy chain variable region polypeptide or a
30 LM609 grafted light chain variable region polypeptide, wherein the $\alpha_v\beta_3$ binding affinity of said high affinity LM609 grafted antibody is enhanced.

114

75. The high affinity LM609 grafted antibody
of claim 74, wherein said functional fragment is selected
from the group consisting of Fv, Fab, F(ab)₂ and scFV.

76. The high affinity LM609 grafted antibody
5   of claim 74, wherein said CDR having at least one amino
acid substitution is selected from the group consisting
of V$_H$ CDR1, V$_H$ CDR2, V$_H$ CDR3, V$_L$ CDR1, V$_L$ CDR2 and V$_L$ CDR3.

77. The high affinity LM609 grafted antibody
of claim 76, wherein said high affinity LM609 grafted
10 antibody comprises the combination of CDRs selected from
the group consisting of:

the V$_L$ CDR1 referenced as SEQ ID NO:82 and the
V$_H$ CDR3 referenced as SEQ ID NO:68;
the V$_L$ CDR1 referenced as SEQ ID NO:82, the V$_H$
15 CDR2 referenced as SEQ ID NO:56 and the V$_H$ CDR3 referenced
as SEQ ID NO:68;
the V$_L$ CDR1 referenced as SEQ ID NO:82, the V$_H$
CDR2 referenced as SEQ ID NO:56 and the V$_H$ CDR3 referenced
as SEQ ID NO:72;
20   the V$_L$ CDR1 referenced as SEQ ID NO:82, the V$_H$
CDR2 referenced as SEQ ID NO:56 and the V$_H$ CDR3 referenced
as SEQ ID NO:70;
the V$_L$ CDR1 referenced as SEQ ID NO:82 and the
V$_H$ CDR3 referenced as SEQ ID NO:72;
25   the V$_L$ CDR3 referenced as SEQ ID NO:86, the V$_H$
CDR2 referenced as SEQ ID NO:56 and the V$_H$ CDR3 referenced
as SEQ ID NO:68;
the V$_L$ CDR3 referenced as SEQ ID NO:90, the V$_H$
CDR2 referenced as SEQ ID NO:56 and the V$_H$ CDR3 referenced
30 as SEQ ID NO:94;
the V$_L$ CDR3 referenced as SEQ ID NO:90 and V$_H$
CDR3 referenced as SEQ ID NO:68;

the V_L CDR3 referenced as SEQ ID NO:90, the V_H CDR2 referenced as SEQ ID NO:56 and V_H CDR3 referenced as SEQ ID NO:68;

the V_L CDR1 referenced as SEQ ID NO:82, the V_H

5 CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced as SEQ ID NO:94;

the V_L CDR3 referenced as SEQ ID NO:90, the V_H CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced as SEQ ID NO:96;

10 the V_L CDR3 referenced as SEQ ID NO:90 and the V_H CDR3 referenced as SEQ ID NO:94;

the V_L CDR3 referenced as SEQ ID NO:90 and the V_H CDR3 referenced as SEQ ID NO:98; and

the V_L CDR3 referenced as SEQ ID NO:90, the V_H

15 CDR2 referenced as SEQ ID NO:56 and the V_H CDR3 referenced as SEQ ID NO:100.

78. A nucleic acid encoding an enhanced LM609 grafted antibody, or a functional fragment thereof, exhibiting selective binding affinity to $\alpha_v\beta_3$ comprising

20 at least one amino acid substitution in one or more CDRs of a LM609 grafted heavy chain variable region polypeptide or a LM609 grafted light chain variable region polypeptide, wherein the $\alpha_v\beta_3$ binding affinity of said enhanced LM609 grafted antibody is maintained or

25 enhanced.

79. A nucleic acid encoding a high affinity LM609 grafted antibody, or a functional fragment thereof, exhibiting selective binding affinity to $\alpha_v\beta_3$ comprising at least one amino acid substitution in one or more CDRs

30 of a LM609 grafted heavy chain variable region polypeptide or a LM609 grafted light chain variable region polypeptide, wherein the $\alpha_v\beta_3$ binding affinity of said high affinity LM609 grafted antibody is enhanced.

1 / 13

```
CAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTT GTG CAG CCT GGA AGG      48
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1               5                  10                  15

TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT      96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
                        20                  25                  30

GAC ATG TCT TGG GTT CGC CAG GCT CCG GGC AAG GGT CTG GAG TGG GTC     144
Asp Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
            35                  40                  45

GCA AAA GTT AGT GGT AGT GGT GGG AGC ACC TAC TAT TTA GAC ACT GTG     192
Ala Lys Val Ser Gly Ser Gly Gly Ser Thr Tyr Tyr Leu Asp Thr Val
        50                  55                  60

CAG GGC CGA TTC ACC ATC TCC AGA GAC AAT AGT AAG AAC ACC CTA TAC     240
Gln Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
    65                  70                  75                  80

CTG CAA ATG AAC TCT CTG AGA GCC GAG GAC ACA GCC GTG TAT TAC TGT     288
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
        85                  90                  95

GCA AGA CAT AAC TAC GGC AGT TTT GCT TAC TGG GGC CAA GGG ACT ACA     336
Ala Arg His Asn Tyr Gly Ser Phe Ala Tyr Trp Gly Gln Gly Thr Thr
            100                 105                 110

GTG ACT GTT TCT AGT                                                 351
Val Thr Val Ser Ser
            115
```

FIG. IA

```
GAG ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT CTC AGC CCA GGA    48
Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
 1           5              10               15

GAA AGG GCG ACT CTT TCC TGC CAG GCC AGC ATT AGC AGC AAC CAC        96
Glu Arg Ala Thr Leu Ser Cys Gln Ala Ser Ile Ser Ser Asn His
             20              25              30

CTA CAC TGG TAT CAA CAA AGG CCT GGT CAA GCC CCA AGG CTT CTC ATC   144
Leu His Trp Tyr Gln Gln Arg Pro Gly Gln Ala Pro Arg Leu Leu Ile
             35              40              45

AAG TAT TCC CAG TCC ATC TCT GGG ATC CCC GCC AGG TTC AGT GGC       192
Lys Tyr Ser Gln Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly
     50              55              60

AGT GGA TCA GGG ACA GAT TTC ACC CTC ACT ATC TCC AGT CTG GAG CCT   240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
 65              70              75              80

GAA GAT TTT GCA GTC TAT TAC TGT CAA CAG AGC AGC GGC AGC TGG CCT CAC  288
Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Ser Gly Ser Trp Pro His
             85              90              95

ACG TTC GGA GGG GGG ACC AAG GTG GAA ATT AAG                        321
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
             100             105
```
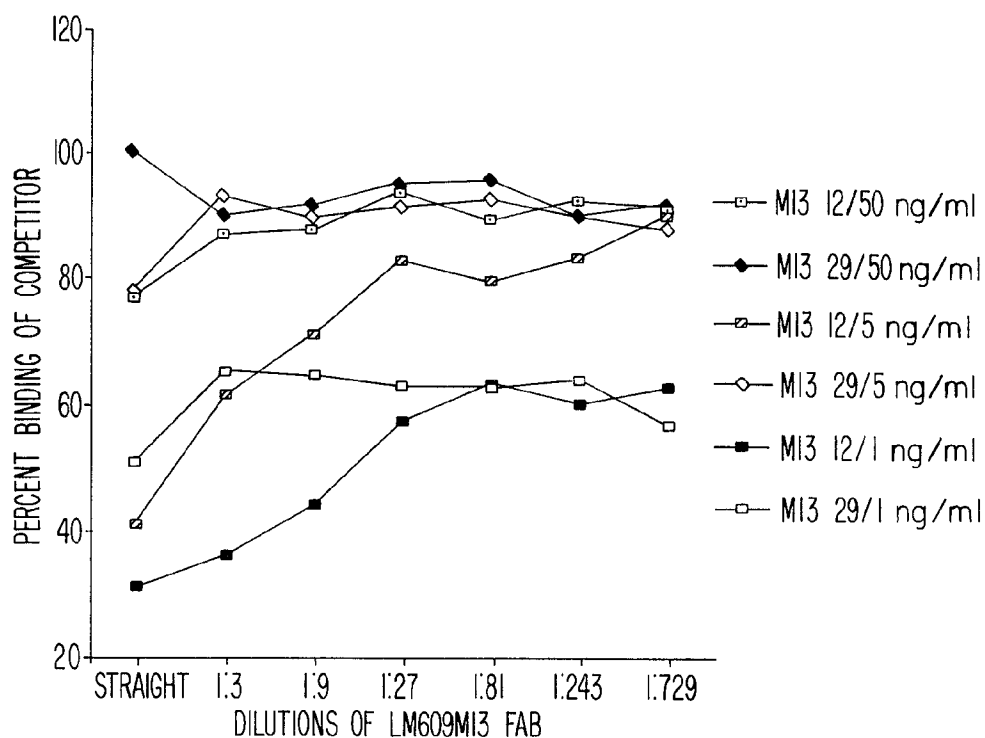
FIG. IB

```
GAA GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA AGG    48
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Arg
 1           5              10              15

TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC GCT TTC AGT AGC TAT    96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Ala Phe Ser Ser Tyr
              20              25              30

GAC ATG TCT TGG GTT CGC CAG ATT CCG GAG AAG AGG CTG GAG TGG GTC   144
Asp Met Ser Trp Val Arg Gln Ile Pro Glu Lys Arg Leu Glu Trp Val
     35              40              45

GCA AAA GTT AGT GGT GGG AGC ACC TAC TAT TTA GAC ACT GTG           192
Ala Lys Val Ser Gly Gly Ser Thr Tyr Tyr Leu Asp Thr Val
     50              55              60

CAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTA TAC   240
Gln Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65              70              75              80

CTG CAA ATG AGC AGT CTG AGA GAC GAG GAC ACA GCC ATG TAT TAC TGT   288
Leu Gln Met Asn Ser Leu Arg Asp Glu Asp Thr Ala Met Tyr Tyr Cys
              85              90              95

GCA AGA CAT AAC TAC GGC AGT TTT GCT TAC TGG GGC CAA GGG ACT CTG   336
Ala Arg His Asn Tyr Gly Ser Phe Ala Tyr Trp Gly Gln Gly Thr Leu
              100             105             110

GTC ACT GTC TCT GCA                                               351
Val Thr Val Ser Ala
     115
```

FIG. 2A

```
GAT ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT GTG ACA CCA GGA      48
Asp Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Val Thr Pro Gly
  1               5                  10                  15

GAT AGC GTC AGT CTT TCC TGC CAG GCC AGC CAA AGT ATT AGC AAC CAC      96
Asp Ser Val Ser Leu Ser Cys Gln Ala Ser Gln Ser Ile Ser Asn His
                 20                  25                  30

CTA CAC TGG TAT CAA CAA AAA TCA CAT GAG TCT CCA AGG CTT CTC ATC     144
Leu His Trp Tyr Gln Gln Lys Ser His Glu Ser Pro Arg Leu Leu Ile
         35                  40                  45

AAG TAT CGT TCC CAG TCC ATC GGG TCT GGG TCC AGG TTC AGT GGC TCA     192
Lys Tyr Arg Ser Gln Ser Ile Gly Ser Gly Ser Arg Phe Ser Gly Ser
     50                  55                  60

AGT GGC ACA TCT GCT CTC AGT ATC CCC TCC AAC AGT GTG GAG ACT         240
Ser Gly Thr Ser Ala Leu Ser Ile Pro Ser Asn Ser Val Glu Thr
 65                  70                  75              80

AGT GGC TCA GGG ACA GAT TTC GGA ATG TTC TAT TGT CAA CAG TGG CCT CAC 288
Ser Gly Ser Gly Thr Asp Phe Gly Met Phe Tyr Cys Gln Gln Trp Pro His
                         85                  90                  95

ACG TTC GGA GGG GGG ACC AAG CTG GAA ATT AAG                         321
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
         100                 105
```

FIG. 2B

FIG. 3
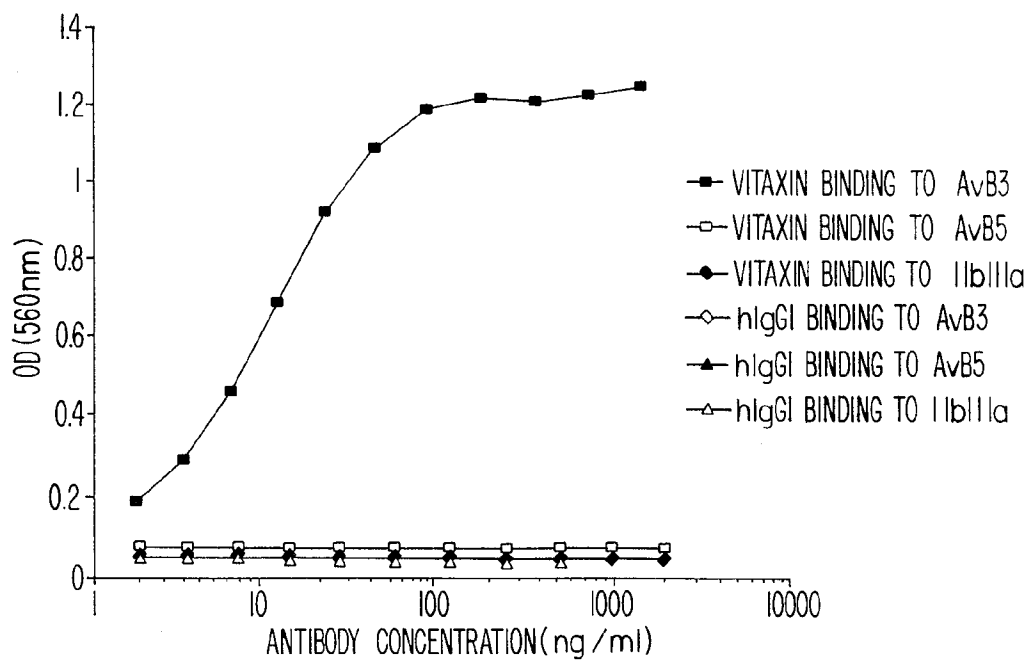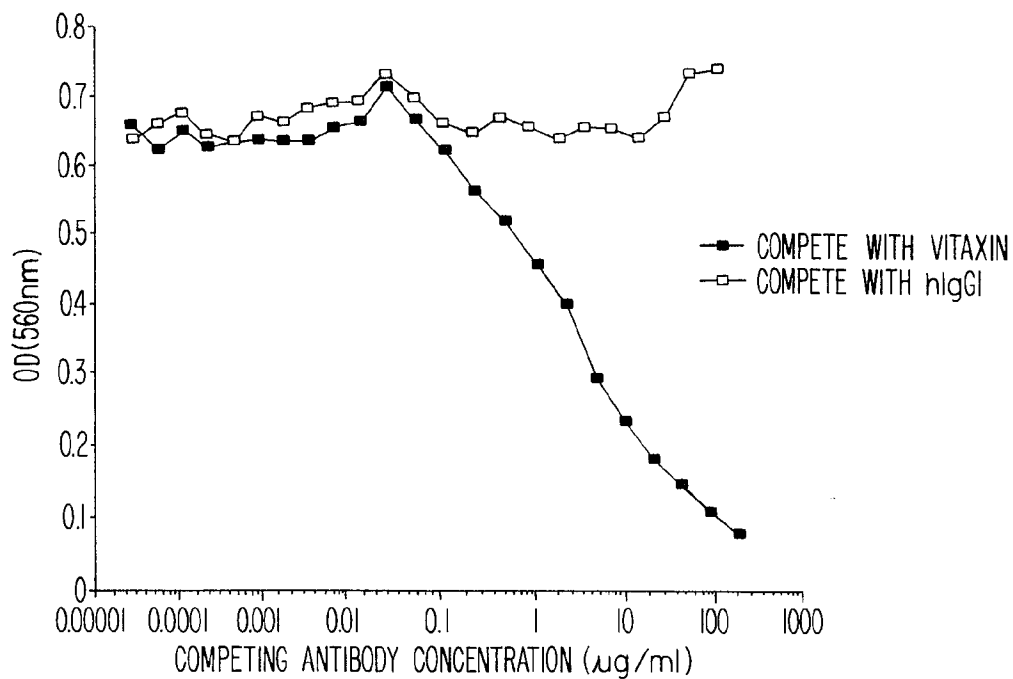

FIG. 4A

FIG.4B

FIG.4C

7 / 13

FIG. 5A

FIG. 5B

CHANGE IN TUMOR WEIGHT (mgs)

REGRESSIONS

Fg/VITAXIN 5/8
Fg/IgGI 1/7
HEp-3/VITAXIN 5/7
HEp-3/IgGI 1/8

VITAXIN   IgGI   VITAXIN   IgGI

Fg TUMOR   HEp-3 TUMOR

TREATMENT (300 μg)

FIG. 6A

MEAN TUMOR WT. (gms)

P VALUE

0.032

0.111

PBS   5   1

VITAXIN DOSE (mg/kg)

FIG. 6B

FIG. 6C

```
GAG ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT CTC AGC CCA GGA
Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly    48
 1               5                  10                 15

GAA AGG GCG ACT CTT TCC TGC CAG GCC AGC CAA AGT ATT AGC AAC CAC
Glu Arg Ala Thr Leu Ser Cys Gln Ala Ser Gln Ser Ile Ser Asn His    96
            20                  25                 30

CTA CAC TGG TAT CAA CAA AGG CCT GGT CAA GCC CCA AGG CTT CTC ATC
Leu His Trp Tyr Gln Gln Arg Pro Gly Gln Ala Pro Arg Leu Leu Ile   144
        35                  40                 45

CGT/ATG TAT CGT TCC CAG TCC ATC TCT GGG ATC CCC GCC AGG TTC AGT GGC
Arg/Met Tyr Arg Ser Gln Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly   192
        50                  55                 60

AGT GGA TCA GGG ACA GAT TTC ACC ATC AGT TCC CTG GAG CCT
Ser Gly Ser Gly Thr Asp Phe Thr Ile Ser Ser Leu Glu Pro   240
65                  70                 75              80

GAA GAT TTT GCA GTC TAT TGT CAA CAG AGT GGC AGC TGG CCT CAC
Glu Asp Phe Ala Val Tyr Cys Gln Gln Ser Gly Ser Trp Pro His   288
        85                  90                 95

ACG TTC GGA GGG GGG ACC AAG GTG GAA ATT AAG
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys   321
            100                 105
```

FIG. 7

FIG.8

(1) WILD TYPE
(2) F^{32}
(3) N^{92}
(4) L^{96}

LIGHT CHAIN
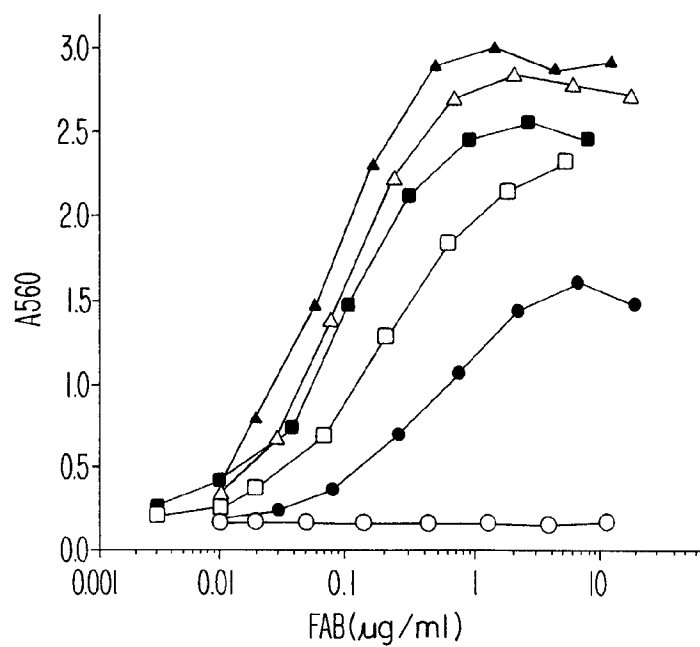
HEAVY CHAIN

p^{60}

Y/H^{97}
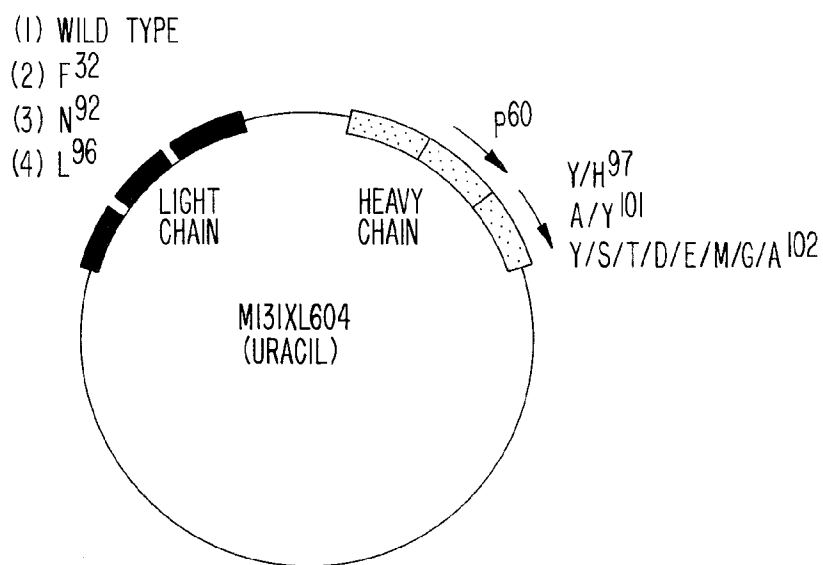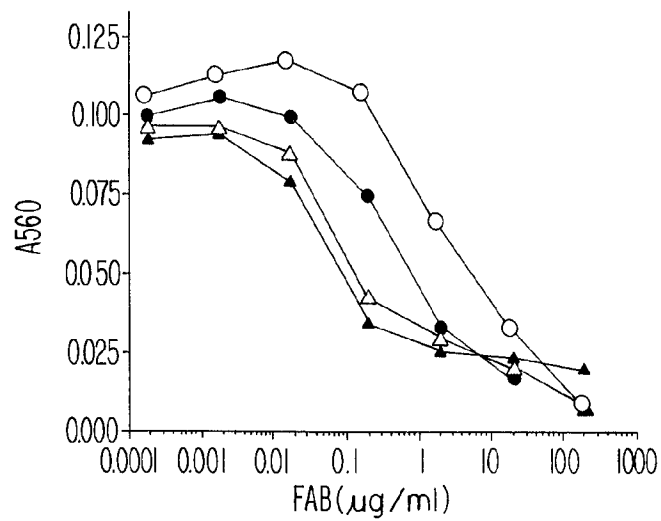A/Y^{101}
$Y/S/T/D/E/M/G/A^{102}$

M13IXL604
(URACIL)

FIG.9

12 / 13

FIG. IOA

FIG. IOB

13 / 13

FIG. 11